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BONE MARROW
CELL PROLIFERATION
IN HEALTH AND
IN HAEMATOLOGICAL DISEASE
DURING CHILDHOOD

BY KARL MARTIN LUNDMARK

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1966

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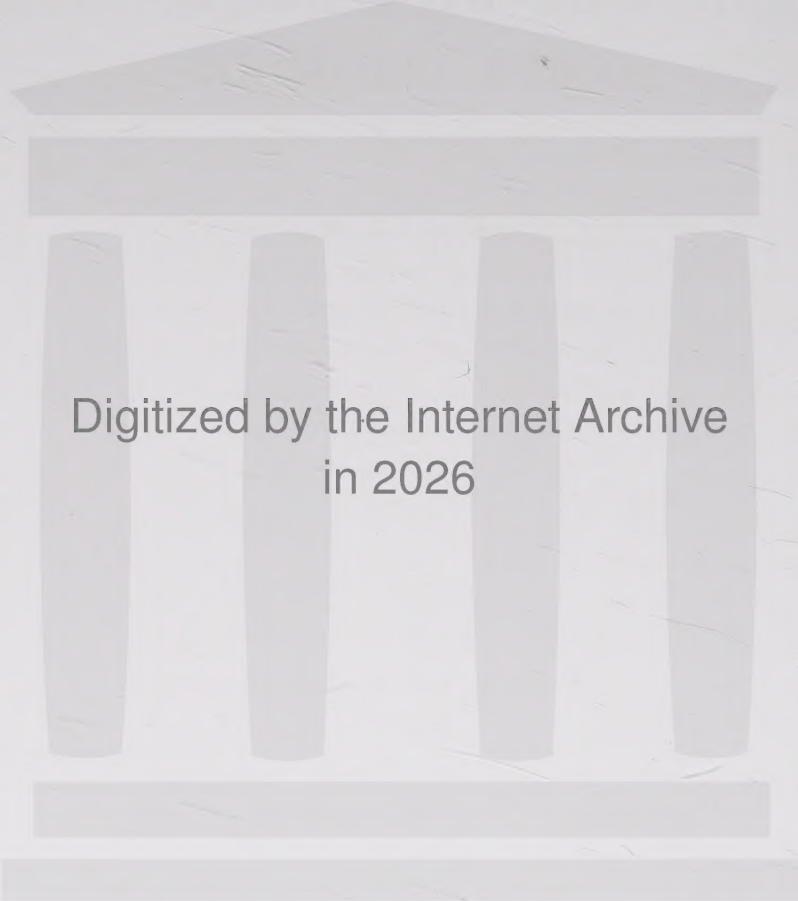
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ACTA PAEDIATRICA SCANDINAVICA

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University of Uppsala, Sweden*

**BONE MARROW CELL PROLIFERATION
IN HEALTH AND IN HAEMATOLOGICAL DISEASE
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By

Karl Martin Lundmark

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Introduction

It is well known that in infants and children the peripheral blood picture changes in a characteristic manner with increasing age. The associated changes in the formation, life span and/or destruction of blood cells are

not fully analysed. The aim of this investigation was to study the cellular kinetics of the bone marrow of both healthy infants and children and those with haematological diseases.

CHAPTER I

Choice of method

The formation of blood cells in the bone marrow

All bone marrow cells are in some phase of a *generation cycle*. After their formation as a result of mitosis (M phase), the cell cycle of the two daughter cells begins with a postmitotic resting or gap period, G_1 . To divide mitotically, a cell must duplicate its chromosomal content. New deoxyribonucleic acid (DNA) is synthesized in the nucleus during the S period or phase which lasts 6–8 hours in most mammalian cells [21]. This is followed by a premitotic resting or gap period, G_2 , of 1–4 hours' duration, which ends in mitosis, M, a process taking 30–70 min. The duration of the G_1 period varies greatly in different cell populations from about one hour to as much as several weeks [35]. The term ' G_0 phase' was introduced to indicate an indefinite prolongation of the G_1 phase, the so called 'no cell cycle' state of potentially proliferative cells, i.e. the phase from which these cells are triggered into the generative cell cycle ($G_0 \rightarrow G_1 - S - G_2 - M$) [67, 88]. A non-proliferative cell is defined as a cell that has not the capacity to synthesize DNA.

A model of bone marrow cell kinetics is outlined in Fig. 1.

Before the different kinds of mature blood cells leave the bone marrow, their precursors have progressed through various stages of *maturation*, which are classified by their cytologic characteristics into a number of serial *compartments*. The *differentiated pool* is constituted by cells with definite characteristics of a differentiated cell line. The bone marrow cells proliferate by mitoses, which define the boundaries between the *generations* of a cell line. This *proliferation* occurs only in compartments containing cells of a more immature type, which constitute a *proliferating pool*. Thereafter the cells pass through some further stages of maturation without division and these cells constitute a *non-proliferating pool*. Cell maturation is a continuous process and the transition from one compartment to another will be gradual. As is indicated in the model scheme of Fig. 1, cytologic boundaries and generation changes do not necessarily coincide.

The very first stage at least, the undifferentiated '*stem cell*' compartment, must be self-perpetuating. As-

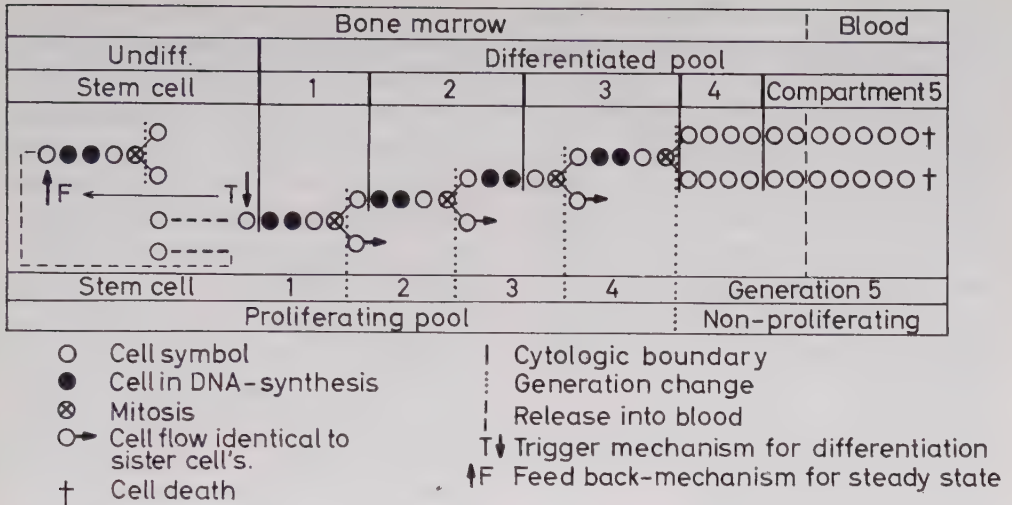


Fig. 1. A schematic outline of the cellular kinetics of the bone marrow. The cell flow is indicated by cell symbols, each indicating one population unit and one time unit. Cellular proliferation occurs by mitoses, which is preceded by DNA synthesis and results in duplication of the cell flow; only one of the sister cells is subsequently followed. The release of cells from the marrow is indicated at the top of the figure. The morphological classification of haematopoietic cells into undifferentiated ('stem cell') and differentiated pools and compartments is also indicated above, while their subdivision into proliferating and non-proliferating pools is indicated below, together with the consecutive generations. It should be noted that the cytologic boundaries and generation changes are not drawn coincident except for the boundary between the proliferating and nonproliferating pools where there is evidence that this is the case [64]. The 'stem cell' pool is self-perpetuating; the transformation of a 'stem cell' to a differentiated cell induces another 'stem cell' divide, thereby maintaining the size of the pool.

suming that cells do not die in this compartment, on the average half of the cells are destined for differentiation, and the other half must remain in the compartment in order to maintain the population in a steady state. A feed-back mechanism is tentatively illustrated in Fig. 1. When a 'stem cell' is triggered off into the differentiated pool, another 'stem cell' is induced to divide, thereby maintaining the size of the 'stem cell' pool. This 'stem cell' compartment has not been identified morphologically, and whether the 'stem cell' is a single entity or several

different cells is still controversial [73, 33].

Defined rigorously, proliferation corresponds to the flow of cells through the serially linked compartments, i.e. the number of cells of a given age produced per unit time. An increase of the production of blood cells can occur if (1) more 'stem cells' differentiate into differentiated cell lines, or (2) the number of mitoses within the differentiated cell lines increases. With these conditions reversed, a decrease of the production will result. Under pathological conditions

other factors, such as altered cell viability, may influence cell production. The production is probably regulated by feed-back mechanisms whereby a lack of a circulating special cell type stimulates the bone marrow to produce and deliver new cells of the deficient type and *vice versa*. There is evidence that humoral factors affect the proliferation and maturation of haemopoietic cells [104].

The estimation of bone marrow cell proliferation

Cell production can be characterized by determining parameters that define changes in the proliferative activity of cells capable of division. Such a characterization is dependent upon a correct differential cell analysis because of the heterogeneity of the bone marrow cell population. The interrelationship between generation change (mitosis) and cytologic change (compartment boundary) must be considered, as must also the problems of the connection between the differentiated cell lines and one or more 'stem cell' compartments.

Differential count. The distribution of a cell type calculated as a percentage is primarily dependent upon both the proliferative activity of the preceding cell types, i.e. the influx into the compartment, and also the time that the actual cell remains cytologically unchanged, i.e. compartment transit time. In a proliferating pool the cell flow is doubled on each division (Fig. 1). This flow increase is usually reflected in the differential count by a successive in-

crease in the proportions of consecutive compartments. Calculations of cell flows from such a distribution pattern necessitate, however, knowledge of the interrelationship between cytologic boundaries and generation changes and also of transit times. Differential counts alone, therefore, do not usually yield sufficient information about cellular proliferation.

Mitotic count. By supplementing differential counting with a mitotic count, the *mitotic index*, i.e. the proportion of cells of a particular type in mitosis, can be calculated; this is a valuable parameter of cellular proliferation. Mitosis itself occupies only a short part of the total generation time. Therefore, relatively few mitoses are to be seen at any instant in a proliferating compartment, and a high number of cells have to be counted to get reliable figures. Furthermore, it may be difficult to identify early prophase and late metaphase and to classify the cell type undergoing mitosis. In ordinary bone marrow smears the mitotic index is usually underestimated [57]. More correct figures are obtained with squash preparations, where, however, the mitotic figures cannot be assigned to their specific cell lines [64].

DNA labelling. Cell division is preceded by DNA synthesis. During recent years isotopically labelled, specific *DNA precursors* have become available. These are incorporated into new DNA during cellular proliferation and can be detected in individual cells. Once in the phase of DNA synthesis, a cell is destined to undergo mitosis

[35]. The *labelling index* is the proportion of labelled cells in a given cell population and is a relative measure of cellular proliferation, determining the proportion of cells in DNA synthesis during the incubation period. In principle, the proportion of cells with labelled DNA is proportional to the mitotic count, each indicating different phases in cellular replication [70]. The much longer duration of the S phase, as compared with mitosis, gives higher and therefore more reliable figures when it is used as a marker. The cell classification problems are also much simplified since it is much easier to classify a non-mitotic cell.

If one cell generation could be defined morphologically, its labelling index would correspond to the ratio between duration of S phase and generation time. The size of a differentiated, proliferating pool is regulated by (1) the influx from the 'stem cell' pool, (2) the number of successive mitoses, and (3) the generation transit times. The labelling index of such a pool can be used to extract information concerning the kinetics of the pool; this will be discussed later.

DNA labelling can be used in both *in vivo* and *in vitro* experiments. Because of the hazards of radiation damage from intranuclear radioisotopes, the use of labelled DNA precursors in human tissues is confined mainly to *in vitro* methods [32].

Bone marrow cell culture. Cellular proliferation *in vitro* can be estimated by cell counts, mitotic counts or DNA labelling. The present technical methods of bone marrow cell culture

have certain important drawbacks which become more pronounced the longer the culture is followed [116].

Treatment of a cell culture with colchicine causes a gradual increase in mitotic index and has been used to analyze the proliferative potentials of bone marrow cells [7]. However, a long-term culture must be studied, and the knowledge of the mechanisms of colchicine action is incomplete.

When bone marrow cells are incubated for a *short term* with a labelled DNA precursor, no changes in morphology, behaviour or chemistry can be observed [116]. Such an experiment does not correspond, strictly speaking, to a cell culture where because of the longer duration, changes in the cells are manifest. The short-term experiment therefore minimizes the limitations of the cell culture method and the advantages of DNA labelling can be utilized more fully.

When estimating the capability of individual bone marrow cells to incorporate DNA precursors, the cellular composition of the marrow specimen is also determined. The use of this method yields information about both proliferation and maturation of bone marrow cells. It therefore seemed a suitable method for the present investigation.

Short-term incubation with labelled DNA precursor

DNA precursor. The most widely used specific DNA precursor is *thymidine*, which is a nucleoside, consisting of the pyrimidine base thymine and deoxyribose. Cells in S phase syn-

thesize DNA into which they will incorporate thymidine after phosphorylation to thymidine-triphosphate. The measurable duration of labelled thymidine in DNA is longer than 10 hours [55]. The labelled DNA can be traced to the next generations, diluted by unlabelled, newly synthesized DNA in successive cell divisions.

Isotope. Thymidine can be labelled with tritium (^3H) which is a radioactive isotope of hydrogen (^1H). Tritium with a half life of 12.5 years emits low energy beta particles whose range in tissue is about $1\ \mu$ [25]. The initial uptake of thymidine is not dependent on the position of the tritium atom in the thymidine molecule [29] and is the same when radioactive carbon is used as the label [41].

Detection of labelled material. Labelled material in cells may be detected by *autoradiography*. The general principles of autoradiography are simple: Decaying atoms of a radioisotope within a cell emit ionizing particles which sensitize silver halide crystals in a nearlying photographic emulsion which can be reduced to metallic silver (grains) in the presence of a developer [18, 25]. The level of cellular detail to which these grains can be localized is limited by the resolution of the photographic emulsion and the energy of the beta particles.

Estimation of the incorporation of label. With the autoradiographic method the two parameters used in estimating the ^3H -thymidine incorporation are the number of developed silver grains over the individual cell nucleus, i.e. the *grain count*, and the proportion of labelled cells in a given cell population, i.e. the *labelling index*.

Time of incubation. Bone marrow cells become maximally labelled with respect to both grain count and labelling index after approximately 40 min incubation with ^3H -thymidine according to Rubini et al. [95]. The labelling data obtained after one hour's incubation of cell suspensions with ^3H -thymidine approximate those obtained in corresponding tissues *in vivo* one hour after the injection of ^3H -thymidine [59, 71, 93].

Short-term incubation with ^3H -thymidine yields valuable information about cellular proliferation without too great technical difficulties [94, 117]. The problems of the effects of radiation and reutilization are of minor importance [8]. The one-hour incubation period has become a standardized procedure when estimating *in vitro*-labelling parameters [95]. This method will be described and the interpretation of data obtained will be discussed in the next chapter.

CHAPTER II

Methods

A. DESCRIPTION OF METHODS

Preparation of autoradiograms

Sterile equipment was used in all procedures until after the incubation. Cell suspensions were handled under aseptic conditions. All glassware was silicone-coated with Desicote Beckman®.

Bone marrow aspiration. The bone marrow samples were obtained by tibial puncture in children up to 18 months of age and by puncture of the posterior spine of the iliac crest in older children. Local anaesthesia (Xylocaine® 1%) was always used. A few of the older children were sedated with chloral hydrate rectally prior to the puncture. All samples except those from newborn infants were obtained between 9–10 a.m.

Bone marrow was aspirated with Franzén's bone marrow aspiration set (Kifa, Stockholm) which consists essentially of a Luer-Lok syringe equipped for single-handed use, together with a stainless steel needle, 1.4 or 1.8 mm in outer diameter [30]. The aspiration set was rinsed with 1% MgK₂EDTA-solution (Merck, Germa-

ny) before use, leaving only a few drops in the syringe. The aspirated volume was 1.0–1.5 ml, usually obtained in one aspiration. Immediately thereafter, the bone marrow was transferred to an EDTA-rinsed glass tube, which was shaken gently. These precautions were usually enough to prevent coagulation. However, when repeated aspirations were necessary coagulation did become a problem.

Within 10–15 minutes after the aspiration, 1.0 ml was transferred to a new vessel for incubation with ³H-thymidine. In a few cases less than 1.0 ml was aspirated and incubated.

Incubation. The technique used in preliminary experiments for the incubation of bone marrow cells with ³H-thymidine was that used by Bryant [19]. It was later modified partly in accordance with the procedure of the Brookhaven group [94].

Tritiated thymidine with a specific activity of 3.0 C per millimole was used (Schwarz BioResearch Inc., Orangeburg, New York). A stock solution of 500 μ C per ml was diluted with 0.9 per cent NaCl to 10 μ C per ml.

All ^3H -thymidine was stored at -20°C . Diluted solutions were discarded after two months.

^3H -thymidine was added to the bone marrow cell suspension to give a final concentration of $1.0\ \mu\text{C}$ per ml. No regard was taken of the varying content of nucleated cells per mm^3 in the cell suspensions from different cases. The suspension was incubated at room temperature ($23\text{--}24^\circ\text{C}$) in a 4 ml covered porcelain beaker, which was gently shaken every fifth minute.

Preparation of smears. After incubation for one hour, smears were made on gelatin coated microslides, using an artist's brush, sable hair 00 [20]. The brush was rinsed in polyvinylpyrrolidone (PVP, Peristone®, Bayer), and the excess of PVP was absorbed on a lens tissue paper. The brush was then dipped into the bone marrow cell suspension and a streak was gently 'painted' on the slide. One dip was used for two streaks by rotating the brush 180° . Four streaks were 'painted' on each slide. After every fifth slide the brush was rinsed in PVP and the incubation vessel gently shaken. At least twenty slides were prepared from each incuba-

tion specimen. The smears were rapidly air dried after 'painting' and then fixed in absolute methanol for two hours with one change.

Autoradiography. Autoradiograms of the slides were prepared using Kodak AR. 10 stripping film as according to Pelc [86]. The mounted slides were air dried for two hours and then stored at 4°C in light tight boxes with silica gel as a desiccant. After 12 days' exposure the slides were developed in Kodak D. 19 B for 5 minutes, rinsed in distilled water for 10–20 seconds, fixed in Kodak F 52 acid fixer for 10 minutes and finally rinsed in tap water for 30 minutes which was changed after 15 minutes (temperature $18\text{--}19^\circ\text{C}$). The excess film was trimmed away and the slides were dried in front of a fan.

When the slides were covered with stripping film within 2–3 weeks after the smears were made, some of them were spoilt by "bubbles" which appeared along the streaks after developing. By first passing the streaks through decreasing concentrations of ethanol (absolute, 70%, and 50%, with 5 minutes in each) and then

Fig. 2. (opposite page) Colour photomicrographs of bone marrow cells. Those indicated below with [F] were taken through the stripping film used for autoradiography; labelled cells are indicated, as such, below. The granulocytes depicted are all neutrophils. *May-Grünwald-Giemsa stain $\times 1,000$.*

a) 'Stem cells', b) 'Stem cell' (lower right), metamyelocyte (above) and polymorphonuclear granulocyte, c) [F] 'Stem cell', d) [F] 'Stem cell' (below) and lymphocyte, e) [F] 'Stem cell' (above), polymorphonuclear granulocyte (lower right) and lymphocyte, f) [F] Labelled 'stem cell', (g) [F] Labelled 'stem cell' (above) and labelled myelocyte, h) Lymphocyte, i) Proerythroblast (right) and lymphocyte, j) Proerythroblast (below) and orthochromatic erythroblast, k) Proerythroblast (above) and polychromatic erythroblast, l) Basophil (above) and orthochromatic erythroblast, m) Basophil (middle), polychromatic erythroblast (left) and lymphocyte, n) [F] Polychromatic (above) and two labelled basophil erythroblasts, o) Promyelocyte (above) and polymorphonuclear granulocyte, p) Promyelocyte, q) Myelocyte (above) and lymphocyte, r) Metamyelocyte, s) [F] Labelled promyelocyte, t) [F] Labelled promyelocyte, u) [F] Labelled myelocyte, v) Immature monocyte, x) [F] Labelled myelocyte (right) and labelled immature monocyte.

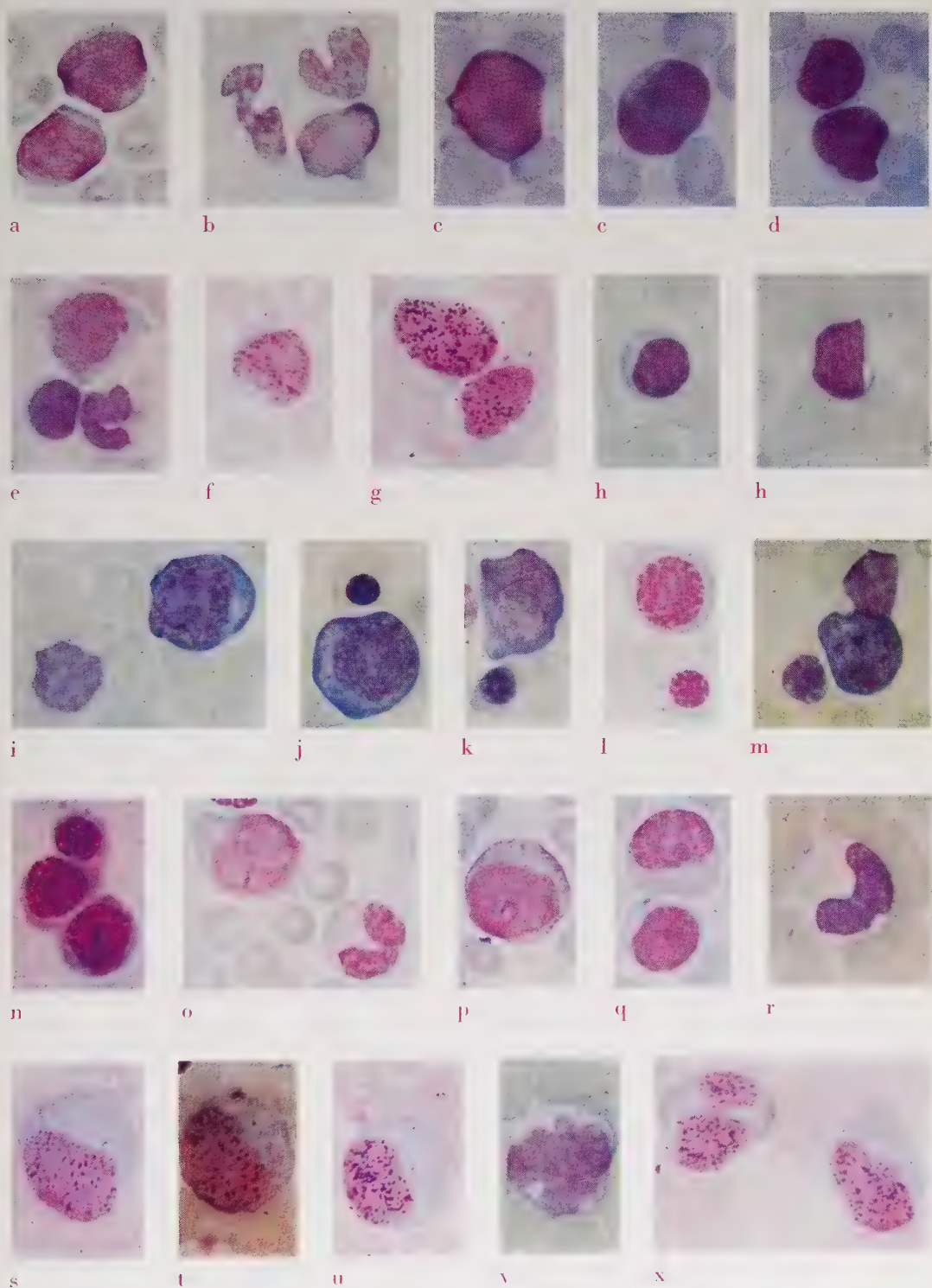


Fig. 2.

distilled water over night before applying the film, bubbles were avoided.

Staining. The streaks were stained with May-Grünwald and Giemsa stains through the developed film. The procedure was as follows: (1) Citric acid-phosphate buffer, pH 5.8, for 3 minutes, (2) 50 % methanol for 3 minutes, (3) absolute methanol for 1 minute, (4) May-Grünwald stain stock solution for 4 minutes, (5) May-Grünwald stain diluted 1:3 with buffer for 2 minutes and then rinsed off with buffer, (6) Giemsa stain for 20 minutes (12 ml Giemsa stock solution, 3 ml absolute methanol, buffer to make up to 100 ml), (7) the stain was carefully rinsed off with buffer and the slides were immediately dried in front of a fan. It was observed during preliminary studies where the initial methanol treatment was omitted that there was in many autoradiograms a reddish granulation of the film and a tendency of the silver grains to fade. The recommendation of Feinendegen and Bond [40] to treat the autoradiograms with buffer and methanol before staining was effective in greatly reducing these effects.

Analysis of autoradiograms

The stained and dried slides were examined microscopically with an oil immersion objective.

In each case investigated, one thousand consecutive nucleated bone marrow cells were counted differentially and the number of silver grains over the individual cell nuclei was counted, as described below. In a satisfactory

autoradiogram one or two streaks were enough to yield 1,000 cells. Additional cells belonging to the 'stem cell' pool and to the proliferating pools within erythropoiesis and granulopoiesis (see below) were counted so as to obtain at least 200 cells from each pool. These counts were made on the same smears as those used for the '1,000-counts'.

Differential cell count

The cells were classified into compartments according to the morphological criteria given by Wintrobe [115] with some modifications as listed below. This classification is based on the changes in the morphological characteristics of nucleus and cytoplasm which are associated with increasing maturation. The following descriptions are of cells stained by the May-Grünwald and Giemsa stains. The colour plates in Fig. 2 are included in order to complement the morphological description given below. They were specially selected to illustrate classification difficulties due to the stripping film procedures and to grains overlying the cells.

'Stem cell' compartment (abbreviated: *St*). The term 'stem cell' is used for immature, proliferative cells which lack the definite characteristics of a differentiated cell line. This means that the 'myeloblast', conventionally placed in the granulopoietic series, is included in this group.

The cell diameter varies from 10–20 μ . The nucleus is rounded and is sometimes slightly indented. The chromatin structure is fine and reticular

and there are, generally, one to four nucleoli visible. The cytoplasm is sparse, moderately basophilic and agranular (Fig. 2 a, b, c, d, e, f, g).

Erythropoietic series. Characteristic features are the round and usually central nucleus, the granular chromatin structure, and the absence of cytoplasmic granules.

(a) *Proerythroblast (Er-1)*. The cell diameter is about $15\ \mu$. The nucleus has a fine-granular pattern and distinct nucleoli. The cytoplasm is relatively sparse, stains deep-blue and often unevenly and may show a perinuclear halo and 'buddings' (Fig. 2 i, j, k).

(b) *Basophilic erythroblast (Er-2)*. The chromatin strands are thick and stain deeply, giving a coarse, granular appearance. Nucleoli are generally not visible. The cytoplasm is basophilic (Fig. 2 l, m, n).

(c) *Polychromatic erythroblast (Er-3)*. The nucleus has irregularly lumped chromatin and no visible nucleoli. The cytoplasm is polychromatic (Fig. 2 k, m, n).

(d) *Orthochromatic (acidophilic) erythroblast (Er-4)*. The nuclear chromatin is very coarse or completely condensed. The cytoplasm of erythroblasts with these nuclear characteristics is acidophilic, as a rule, but sometimes more polychromatic (Fig. 2 j, l).

Proerythroblasts, basophilic and polychromatic erythroblasts constitute the *proliferating* pool (*Er-P*), and orthochromatic erythroblasts the *non-proliferating* pool (*Er-NP*) (cf. Table 9, p. 39). Strictly, the non-proliferating erythropoietic pool comprises not only orthochromatic erythroblasts but also

reticulocytes and erythrocytes. However, by eliminating the non-nucleated erythroid cells a clear cytologic delimitation of the non-proliferating erythroblast pool is obtained. The relative 'cell flow' through the non-proliferating pool can be calculated from the size and transit time of this pool.

The granulopoietic series. Cytoplasmic granules are present from the earliest stage. The nucleus varies in shape and position. The chromatin strands have a meshwork structure.

(a) *Promyelocyte (Gr-1)*. The cell diameter varies from $15\text{--}25\ \mu$. The nucleus is oval, somewhat eccentrically situated and has a fine reticular chromatin structure with distinct nucleoli. The moderately to strongly basophilic cytoplasm may vary in amount from relative sparseness to abundance. The more immature forms contain only azurophilic granules. Later, specific granules appear, usually in a central perinuclear zone (Fig. 2 o, p, s, t).

(b) *Myelocyte (Gr-2)*. The nucleus is slightly indented, has a relatively coarse chromatin structure and no visible nucleoli. The cytoplasm is acidophilic, with abundant specific granules. The cytoplasm of the more immature forms may contain remains of basophilia and some azurophilic granules (Fig. 2 g, q, u, x).

(c) *Metamyelocyte (Gr-3)*. The nucleus is somewhat ellipsoid or kidney-shaped and has a coarse chromatin structure and well marked outline (Fig. 2 b, r).

(d) *Polymorphonuclear granulocytes (band cells and segmented cells, Gr-4)*. The cells are uniform in size and

granule content. The nucleus is rod-like or lobulated (Fig. 2 b, e, o).

The neutrophilic, acidophilic and basophilic series are distinguished by the staining reactions of their granules. The neutrophilic series was classified according to the foregoing groups (a), (b), (c), and (d), while the eosinophils were classified as 'immature' or 'mature', corresponding to (a)-(b) or (c)-(d), respectively. The basophilic granulocytes were all assigned to a single group.

In granulocytopoiesis, promyelocytes and myelocytes constitute the *proliferating* pool (*Gr-P*), and metamyelocytes, band cells and segmented granulocytes the *non-proliferating* pool (*Gr-NP*) (cf. Table 9, p. 39).

In the following discussion of the results of the present study, compartments and pools within the *neutrophilic* granulopoietic cell series will be referred to without the prefix 'neutrophilic'.

Lymphocytes. Most of the lymphocytes in the bone marrow correspond to the '*small lymphocyte*' of the blood. They are small cells 6-12 μ in diameter, with round nuclei containing clumped chromatin. Distinct nucleoli are not visible with standard staining methods [46]. The cytoplasm is sparse, often hardly visible, basophilic and sometimes contains azurophilic granules. The '*large lymphocyte*' is more varied in appearance. It has a diameter as great as 20-25 μ , the nuclear shape varies, and the cytoplasm stains from pale to deep blue. Both types of lymphocyte were assign-

ed to a single group (Fig. 2 d, e, h, i, m, q).

The monocytic series. The indented nucleus, fine and lacy chromatin structure and gray-blue cytoplasm are characteristic of this series. The monocytes are usually rather rare in bone-marrow smears and were separated in sub-groups only when they were increased in number.

(a) *Immature form of monocyte.* The diameter is 15-20 μ . The nucleus is slightly to moderately indented and nucleoli are usually visible. Fine, azurophilic granules appear in later stages of this form (Fig. 2 v, x).

(b) *Monocyte.* The nucleus is convoluted without visible nucleoli. The abundant cytoplasm contains numerous azurophilic granules and sometimes vacuoles and phagocytized material.

Plasma cell. Characteristic are the eccentric circular nucleus and the deep-blue, non-granular cytoplasm with a perinuclear halo. No division into subgroups was made.

The thrombocytopoietic series. This series was not studied as the method used for making smears gave a low and irregular number of recognizable megakaryocytes. Moreover, the megakaryocytes present accumulated at the end of the streak, which was usually not covered with autoradiographic film.

Disintegrated cells. Cellular remnants of disintegrated and unrecognizable cells that had the structure of nucleated cells, were included in the differential counts under the above heading.

Grain count

Background. Developed silver grains whose development is not induced by tritium decay, constitute the background fog which may lead to an overestimate of 'true' labelling. It has been found that the background fog over cells in a section may differ from the background in an area not containing cells [112]. To get, therefore, an estimate of the magnitude of the background fog over the cells, grains were counted over erythrocytes in the bone marrow streaks, since these cells, which were not radioactive, had passed exactly the same technical procedures as the nucleated bone marrow cells. The number of grains over 500 erythrocytes were counted on each slide used for background control. 125 cells were counted on each of the four streaks of a slide, the region counted being changed from streak to streak as shown in Fig. 3. The background fog per $100\mu^2$ was then calculated assuming a mean erythrocyte diameter of 7.2μ .

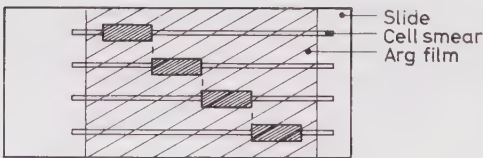


Fig. 3. Estimation of background fog. Within each of the shaded areas 125 erythrocytes were grain counted, i.e. in all 500 erythrocytes on each slide. The background fog was calculated as grains per $100\mu^2$.

Every three months, the background fog of one autoradiogram from each of three cases was estimated, in 21 months a total of 21 slides. The mean background of these control slides was 0.2 grains per $100\mu^2$ (range 0.07–0.5).

Labelled cells. The number of grains over the nuclei of all bone marrow cells counted was determined. The grain count was graded into the following classes: 0, 1, 2, 3, 4, 5, 6–10, 11–15, 16–20, 21–25, 26–30, 31–35, 36–40, and 'more than 40' grains per cell nucleus. A cell was considered labelled when it had 4 grains or more overlying its nucleus (see below).

Labelling index. From the differential counts the proportion of labelled cells of each individual cell type (compartment) and of each proliferating pool can be estimated.

The main proliferating pools in the bone marrow are (1) the 'stem cell' pool, (2) the erythropoietic pool, and (3) the granulocytopoietic pool. In each pool a differential count and grain count of at least 200 cells was used for calculation of the labelling index.

Mean grain count. The mean grain count is the sum of all the grains in the labelled cells divided by the number of labelled cells. It was estimated both for compartments and proliferating pools. In this calculation the grain counts '6–10', '11–15', etc., were taken as the mean value, i.e. 8, 13, etc., and 'more than 40' was assessed as 50 grains.

B. EVALUATION AND DISCUSSION OF THE METHODS

The parameters obtained with the methods used in this study were based upon differential and grain counts of bone marrow cells. There were many sources of error which influenced the final results, and these and the possibilities of reducing them will be discussed later.

Preparation of autoradiograms

Bone marrow aspiration. Some intra-individual variation must be expected as bone marrow is a non-homogeneous tissue. The reduction of this error by multiple punctures in the same individual was not feasible, but the method was standardized with regard to the time, site, and technique of puncture.

In man, most cell systems exhibit diurnal changes in composition and function [6]. For this reason all aspirations were performed at about the same time of day, i.e. 9–10 a.m., throughout the investigation with the exception of newborn infants. During foetal life and in the newborn there is apparently no diurnal rhythm [54]. The time of day at which the aspiration is performed is therefore presumably unimportant in the newborn. Bone marrow specimens obtained from different sites, the tibia in infants and the iliac crest in older children, appear to be quite comparable as to their cellular composition [44, 53]. The volume of marrow necessary for the incubation procedures was about

1.0 ml; with the aspiration set used it was usually possible to obtain this volume in a single aspiration, though this necessitated a certain amount of admixture with peripheral blood. Os-good and Seaman [83] found large variations in the total nucleated cell count of the first 0.1 ml portions while the following 1 ml portions showed comparatively good agreement with a single 1 ml sample, i.e. one 'containing' the initial and variable 0.1 ml sample. They concluded that a 1.0 ml sample was more representative of the tissue than a 0.1–0.2 ml sample. The addition of peripheral blood to a marrow specimen affects mainly the total nucleated cell count, while the differential count is apparently changed only when the marrow is extremely cell poor and/or the peripheral blood has a very high nucleated cell count. Thus, the presence of some peripheral blood is not likely to be important.

There is a tendency for relatively fixed cells, such as megakaryocytes, to remain behind in the marrow at aspiration. This phenomenon resulted in wide variations in the number and distribution of megakaryocytes in the bone marrow sample. These variations could probably be reduced to some extent by further attention to the technique of aspiration.

Incubation. Cell counts before and after incubation were compared in eight cases. The mean difference was -4.1% (range -11% $+$ $+6\%$), and was tested by an analysis of variance

and found not significant ($p > 0.10$). Qualitative changes were tested in the following way. The proportions of erythroblasts and disintegrated cells in 500 cells in streaks made after 30 and 60 minutes incubation were compared in five cases. There was no significant change for either cell type ($p > 0.25$).

Preparation of smears. In order to obtain reproducible results in differential counts of bone marrow cells it is necessary to obtain an even distribution of different cell types in the streak. For this reason the bone marrow incubate was shaken regularly before streaking to keep the sample as homogeneous as possible.

The cell distribution on slides was tested by comparing differential counts of 500 cells on each third of a slide from six different cases, as shown in Fig. 4. For this purpose the streaks were not covered with stripping film in order to facilitate differential counting. The results are given in Table 1. There were no significant variations in the distribution of any cell type along the streaks (analysis of variance) except for megakaryocytes which accumulated at the end of the streak.

Disintegrated cells must be included in the differential count if the correct proportions of other cell types are to be obtained. These cells have been ignored in most bone marrow studies, but when they have been reported their relative numbers have varied considerably. Osgood and Seaman [83] noted 19% disintegrated cells (range 10–30%) and Gairdner et al. [44] found an average of 11% 'unrecognizable smear cells' (range 0–30%).

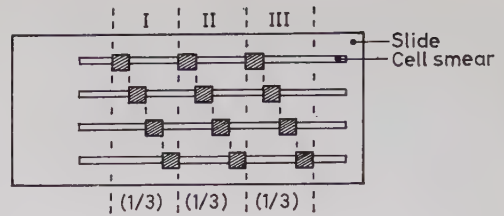


Fig. 4. Cell distribution on different parts of a bone marrow smear. Within each shaded area 125 consecutive nucleated cells were differentiated, in all 500 cells on each third of the test area of the autoradiogram.

In this study, the mean proportion of disintegrated cells in streaks from 53 healthy cases was 4.2%, which is smaller than the figures given above, and the variation was relatively small (standard deviation $\pm 2.6\%$) (cf. Appendix). During preliminary experiments, brush streaking was found to be less damaging to the bone marrow cells than when a coverslip was used. The criteria used for classifying cells as 'disintegrated' were not given in the studies cited above. However, any differences as regards their classification are probably minor and are thus only likely to account for a small part of the difference between the present results and those cited above.

The comparison of cell distributions in brush smears and coverslip smears was difficult, as coverslip smears are usually uneven both in thickness and cell distribution. No systematic comparison was therefore attempted, but when differential counts from even and thin regions of coverslip smears of 5 cases were compared with the counts from brush smears, no striking or consistent differences were found.

TABLE 1. *Cell distribution of 500 nucleated cells on different test areas of bone marrow smears. Cf. Fig. 4.*

Cell type	Case						Case 1—6 pooled	Test area
	1	2	3	4	5	6		
Er—1	1 3 2	1 5 0	2 2 3	1 2 2	2 6 6	3 0 4	10 18 17	I II III
Er—2	2 5 3	10 10 11	5 5 5	1 3 2	6 9 5	1 2 3	25 34 29	I II III
Er—3	7 16 19	37 26 31	16 19 16	3 2 5	13 20 12	4 6 5	80 89 78	I II III
Er—4	10 19 24	54 60 62	31 30 31	5 2 5	23 27 30	7 8 10	130 146 162	I II III
Er	20 43 38	102 101 104	54 56 55	10 9 14	44 62 53	15 16 22	245 287 286	I II III
St	27 46 39	20 13 22	43 22 33	46 51 58	40 51 36	40 46 40	216 229 228	I II III
Gr—1	16 14 17	16 15 16	24 15 16	13 19 21	13 12 21	21 22 19	103 97 110	I II III
Gr—2	37 39 34	41 39 26	49 41 38	20 36 41	25 28 39	28 26 23	200 209 201	I II III
Gr—3	39 27 30	29 30 22	25 22 24	28 30 42	14 8 14	24 23 23	159 140 155	I II III
Gr—4	168 151 154	135 136 157	159 165 143	133 154 161	128 112 127	112 138 131	835 856 873	I II III
Gr	260 231 235	221 220 221	257 243 221	194 239 265	180 160 201	185 209 196	1237 1302 1339	I II III
Eosino- phils	14 16 13	15 8 5	13 16 11	14 11 11	24 12 16	33 17 26	113 80 82	I II III
Mono- cytes	15 19 23	10 13 4	20 17 23	17 17 19	41 31 27	44 40 35	147 137 131	I II III
Lympho- cytes	163 154 163	135 156 142	162 165 156	220 173 135	180 205 170	186 171 185	1046 1024 951	I II III

Analysis of autoradiograms

Differential cell counts

The cell morphology in children did not differ appreciably from that found in normal adult marrows [44, 110]. The autoradiographic and staining procedures influenced to some extent the cytologic characteristics used for cell classification. The film itself took up some stain giving a reddish hue, which masked the cells to some extent, diminishing their staining contrast. The film also probably interferes to some extent with the actual staining of the cells; this was especially important with cytoplasmic granules, which stained rather poorly under the film. An experienced observer could, however, recognize a characteristic light-diffraction effect, indicating the presence of refractile granules in the cytoplasm. Furthermore, cell nuclei appeared less dense in the autoradiograms than after ordinary May-Grünwald and Giemsa staining of bone marrow smears. It was also difficult to decide the chromatin structure of cells with a very high number of developed grains above their nuclei, but such cells were very few.

The differential classification of the most immature precursor cells in the bone marrow is always difficult, since they lack definite morphological characteristics that indicate into which differentiated cell line they would have developed. It was therefore decided to include in a common cell pool all immature, proliferative cells with no definite characteristics of a differentiated cell line. The existence of a primitive

progenitor pool in the bone marrow is very likely, but its morphological and functional characterization has long been the subject of controversy [33]. The term 'stem cell' is preferred here. Morphologically it corresponds roughly to the 'hemocytoblast' of Ferrata and 'lymphoidocyte' of Pappenheim [61].

In this study attention was paid mainly to the 'stem cells' and to the erythropoietic and the granulopoietic series. The cells were classified together in groups corresponding to the proliferating and non-proliferating pools. Such grouping of the bone marrow cells meant that the cytological boundaries delimiting the different pools had to be defined rigorously.

Bone marrow cells within the proliferating pools can divide. If such a cell synthesizes DNA during incubation with ^3H -thymidine, it becomes labelled. This definitely indicates that the cell belongs to a proliferating pool. Only a fraction, however, of these cells in a proliferating pool take up thymidine during an incubation. By careful morphologic study of such labelled cells during the preliminary experiments certain cytologic criteria were established for distinguishing whether non-labelled cells belonged to proliferating or non-proliferating pools. These criteria are included in the description of bone marrow cell morphology given above.

The relative size of a non-proliferating differentiated pool is dependent not only on the influx into the preceding proliferating pool and the number of mitoses in that pool, but also

on the transit time in the non-proliferating pool. If the differentiated pools are cytologically well delimited, changes in the ratios between the relative sizes of the non-proliferating and proliferating pools indicate changes in proliferation and/or maturation. The same arguments can be applied to the serial compartments of a differentiated cell line and it is convenient to express their sizes relative to the size of the very first compartment. This calculation is liable to large errors due to erroneous classification and is only useful when comparing investigations with uniform cytologic criteria.

'Stem cells'. This cell group must be distinguished from the most immature cells within the differentiated cell series and also from bone marrow lymphocytes.

Generally, the type of *nuclear structure* and degree of *cytoplasmic basophilia* appear to be important criteria for these cells. The chromatin structure is finely reticular in the 'stem cell', a little coarser but still reticular in the early promyelocyte, and finely granular in the proerythroblast. The lymphocyte has clumped chromatin with a tendency for peripheral condensation. The 'stem cell' cytoplasm is moderately basophilic, the promyelocyte cytoplasm is noticeably more basophilic and also more evenly blue, while the proerythroblast has a characteristic deep-blue cytoplasm, often unevenly stained (cf. [1]). The lymphocyte cytoplasm varies from pale to deep blue.

Erythropoietic series. The characteristic features of 'stem cells' and proerythroblasts have been described

above. The compartments on each side of the boundary between the proliferating and the non-proliferating pool are polychromatic and orthochromatic erythroblasts, respectively. When passing this boundary a change in nuclear size occurs and it is consistent with the finding that very few nuclei with a diameter below $6-7\mu$ were labelled. When the *chromatin* appears clump-like or regularly condensed, the cell has apparently left the proliferating pool. The change to orthochromasia is actually not so useful a criterion because some non-proliferating cells are still polychromatic.

Granulopoietic series. The characteristic features of 'stem cells' and promyelocytes have been given above. The myelocyte is the last stage to divide. The *nuclear structure* was a good criterion for distinguishing between proliferative and non-proliferative cells, as granulocytes with coarsely grained chromatin and well marked nuclear outline were never found labelled. Changes in nuclear size and form were not so useful.

Lymphocytes. Attempts to characterize a 'lymphoblast' from among the immature undifferentiated blast cells in normal bone marrow smears, using criteria suggested by Undritz [113] and by other investigators, were unsuccessful. All such cells were therefore included in the 'stem cell' pool.

Lymphocytes exhibit large morphologic variations and some may resemble the 'stem cell'. The boundary between the 'stem cell' and lymphocyte was more closely delimited with the aid of DNA labelling. It was assumed

that the lymphocyte does not incorporate ^3H -thymidine in short-term incubation experiments. A careful morphologic examination was therefore made of cells whose classification into 'stem cell' or lymphocyte was difficult to decide. It was possible to distinguish morphologically between two types, one which was never found labelled, the other which was sometimes labelled; labelled cells always had a finely reticular chromatin structure. The former was classified as a lymphocyte, the latter as a 'stem cell'.

Because of different morphological criteria and other considerations, terms such as 'lymphoid cell', 'lymphoid reticulum cell' and 'transitional lymphocyte' have been introduced [61]. They are all associated with the 'stem cell' concept but the terms may be confusing as there is no definite evidence of the connection of the 'stem cells' to the lymphopoietic cell system. On the other hand, a connection between bone marrow 'lymphocytes' and 'stem cells' cannot be excluded.

Errors in differential counts. In addition to difficulties in classification there are other sources of possible errors. As discussed on p. 17 the bone marrow aspirate was obtained by a standardized technique to minimize sampling errors. Thin smears with well separated cells were selected for counting. The brush streak method gave rows of cells which facilitated the analysis. The even distribution of different types on the slide and the low and relatively constant number of disintegrated cells (cf. p. 18) were additional

advantages of the brush method, contributing to reproducibility.

Analysis of the slides was made by a trained technician under supervision of the author. Regular checks were made to ensure that the morphological criteria remained unchanged in order to avoid any systematic variation in the differential counts. The cytologic boundaries between different compartments are potential sources of error due to wrong classification but by using pools instead of individual compartments the number of these boundaries was considerably reduced; the labelling method used facilitated the definitions of the boundaries between proliferating and non-proliferating pools (cf. p. 20). In different bone marrow investigations, the morphological criteria of different compartments and the number of compartments within the differentiated proliferating pools often differ. This may explain the large variations in mean values of the component cell types given by different investigators. It must be noted that the range obtained by individual investigators is also often rather wide [2, 87].

In comparisons between different cases the reliability of the values of the ratios of non-proliferating to proliferating pools and of the proportional distributions of compartments is much dependent on a uniform classification system. By combining the differential counts from all similar cases the errors due to physiological individual variations are reduced.

Finally, there is an unavoidable statistical counting error which is inde-

pendent of all other sources of error in the technique, and can be expressed in terms of the binomial distribution [83]. The standard deviation of the binomial distribution is $\sqrt{\frac{p \cdot q}{n}}$ where p is the proportion of a specific cell type counted, q is the proportion of other cells counted ($p + q = 1$), and n is the total number of cells counted. The relative variation or coefficient of variation due to the binomial error is reduced by increasing p and/or n . In view of other sources of error it is desirable to keep this statistical counting error as low as possible. The numbers of cells in the immature compartments are small and their enumeration thus involves a relatively large uncertainty. To reduce this error the proliferating compartments within each cell line were combined together in a single pool. This also had the advantage of reducing the number of cytologic boundaries. During preliminary experiments p values were seldom found to be less than 0.10 for different pools. With this proportion a relative statistical counting error of 10 % was considered allowable. This necessitated a differential count of about 1,000 cells, i.e. $n = 1,000$. For higher p values the relative statistical counting error is less.

Estimate of error in a differential count. This error was estimated by comparing differential counts on, (1) different parts of the smear on one slide with no covering film, (2) different smears from the same case covered with film, i.e. the variation among different smears, and (3) dif-

ferent smears from the same case with and without film, i.e. the effect of the film on the differential count.

(1) The results of this test are given in Table 1 (p. 19). No significant differences were found for erythropoietic cells, granulopoietic cells or 'stem cells'.

(2) A differential count of 1,000 cells was repeated on different smears covered with film from 9 healthy children. No significant difference was found for erythropoietic cells and granulopoietic cells. For 'stem cells' there was an (almost) significant difference ($p < 0.05$), however.

(3) A differential count of 1,000 cells from 8 healthy children was made on slides both with and without film. No significant difference was found for erythropoietic, granulopoietic, or 'stem' cells.

Test (1) indicated that there was an even cell distribution on each smear and that the differential counting error on smears without film was relatively small. When different smears from one particular case with and without film were compared (test (3)), new errors were introduced depending partly upon the heterogeneity of the bone marrow sample, i.e. between smears made from different dips of the brush, partly because of increased difficulty in classifying some of the cells due to the autoradiographic procedures. However, these errors were not statistically significant with the exception of the case of the 'stem cells' in test (2), where the difference was possibly significant. When the same smears as in test (2) were used for estimating errors in the

calculation of labelling indices (see p. 26) there were no significant differences for any pool between different smears. The labelling index was calculated from 200 consecutive cells belonging to the actual pool, and in this case it does not matter whether the cells are distributed evenly on the slide or not. If errors in classifying the cells had been the main reason for the differences between the relative frequencies of 'stem cells' in different smears of the same patient (test 2), the labelling index of the 'stem cell' pool would not have shown such good reproducibility. An uneven distribution of 'stem cells' on different smears remains as the most probable explanation. This uneven distribution in different smears is probably due to a real heterogeneity in the bone marrow tissue, although it may be influenced also by different technical procedures. It has been observed that some immature cells tend to remain behind in the bone marrow during aspiration since they were relatively more frequent in bone marrow sections than aspirates [92]. The undifferentiated, proliferative 'stem cells' are considered primitive in type and therefore may also have a tendency to remain behind in the marrow at aspiration; this might well increase their frequency variation in bone marrow smears.

From these test data the relative errors in differential counting can be estimated. These values, including the statistical counting error, were approximately: erythropoietic cells and granulopoietic cells 10–15 %, 'stem cells' 25–30 %. Thus differ-

ences between different cases must be large to be statistically significant for clinical materials of reasonable sizes. This applies especially to the 'stem cell' pool.

Grain count

The parameters obtained by the autoradiographic method were the 'labelling index' and the 'mean grain count' of different cell types.

According to the principles of the method given in Chapter I, the predominant cause of the appearance of grains in the film above a cell nucleus is the uptake of ^3H -thymidine, which indicates that the actual cell was synthesizing DNA during part of the incubation period. This incorporation as also its estimation by grain counting may be influenced by factors that affect the 'true' result.

Incorporation of ^3H -thymidine. The detailed mechanism of thymidine incorporation into DNA is not yet known [105]. There exists an endogenous pool of thymidine, probably associated with the DNA reutilization pathway, and even in low concentrations competing exogenous thymidine may influence the DNA synthesis rate [55]. The uptake is also reflected by the magnitude of intracellular pools of other DNA-precursors and the activity of phosphorylating kinases and DNA polymerases within the cell [27]. Different cell populations may have different abilities to degradate thymidine to products that are not used in DNA synthesis. Marsh and Perry [79] found that in leucocytes of normal and leukaemic peripheral blood this degradation see-

med to be correlated to the level of thymidine phosphorylase, but the mechanism of this connection was not elucidated and bone marrow cells were not studied.

Rubini et al. [95] investigated the *in vitro* metabolism of ^3H -thymidine in bone marrow cells. They concluded that the maximal labelling of the cells was obtained with $1\ \mu\text{C}$ ^3H -thymidine per ml cell suspension. Only a small amount of ^3H -thymidine was actually incorporated into new DNA. In the present study, $0.33 \times 10^{-3}\ \mu\text{mole}$ thymidine was added to 1.0 ml marrow suspension; this is a low concentration compared to the endogenous thymidine. According to the data given by Hughes et al. [55] the thymidine concentration used should not introduce any detectable disturbance of physiological conditions.

The average nucleated cell content of incubated bone marrow cell suspensions was 85,000 per c.mm. (range 22,000–217,000) in samples from 53 healthy infants and children. A similar variation in cell density had no effect on the labelling results in the data of Rubini et al. [95].

EDTA has an excellent capacity to reduce non-specific aggregation of nucleated cells and platelets, but according to Lochte et al. [75] even in low concentrations it has a depressant effect on DNA synthesis. After rinsing the aspiration set with EDTA, the bone marrow was exposed to a concentration which was below the critical depressant limit but still sufficiently high to inhibit coagulation.

It has been reported that *in vitro* labelling for one hour corresponds to *in vivo* 'flash labelling' irrespective of whether the *in vitro* labelling took place at room temperature [15, 93] or 37°C [71]. In view of this temperature non-dependence room temperature was used for incubation in the present study.

Detection of ^3H -thymidine. The efficiency of ^3H -autoradiography with AR. 10 stripping film is usually about one developed grain per 20 disintegrations, but values of 100–200 are also cited [24, 80]. Geometric variations between the labelled material and the photographic emulsion, difficulties in classifying labelled cells, and variations of technical procedures may affect the grain count. The staining method used in this study had no measurable effect on the grain count of individual cells, as was shown by allowing the slides to stand in the staining solutions for several hours with repeated grain counting. It appeared, however, that background grains became smaller in size after the staining procedure.

During preliminary experiments it was found that 12 days' exposure of the film covered slides gave suitable grain counts of labelled cells, making it easier to differentiate between background and 'true' labelling (cf. Table 2, p. 28). Moreover, Baserga and Nemeroff [9] have shown that the percentage of cells identifiable as labelled reaches a maximum value from the tenth day onwards.

Estimation of ^3H -thymidine incorporation. 'Significantly labelled cells' were defined in accordance with Levi

et al. [72] as those cells whose grain counts had no more than a 1% probability of being due to background. With the mean background level found in the control slides (0.2 grains per $100 \mu^2$) this condition was satisfied in cells labelled with four or more grains.

The grain count over polymorphonuclear granulocyte nuclei never exceeded the background range. This indicated, firstly, that no stable incorporation of ^3H -thymidine had occurred in these cells and, secondly, that the fixation procedures were effective in removing any unfixed ^3H -thymidine in the cell nuclei.

The labelling index of an individual cell type is calculated from the differential count and grain count of 1,000 bone marrow cells. There are certain limitations, however, regarding the interpretation of this index.

Errors in the calculation of the labelling index. Under the experimental conditions there were very few cells labelled with 3, 4 or 5 grains. It was therefore relatively easy to identify 'labelled' cells, i.e. those cells with four or more grains. As every cell has to be classified, the differential counting error is identical with that discussed above.

Thymidine incorporation indicates a phase of a cell generation. It is not known in general whether the cytologic change that indicates the transition from one compartment to another, coincides with the generation change, i.e. heteromorphogenic mitosis. It is possible that all mitoses are homomorphogenic though the observable transformation may occur very soon

after the formation of the daughter cells. Whether one compartment contains more than one generation is as yet unknown, as is the number of cell generations within the proliferating pools. For these reasons, it may be misleading to use labelling indices of individual compartments in comparison between compartments and in drawing conclusions about cellular kinetics. However, the use of pools instead of compartments simplifies the matter. Cytologic boundaries within the proliferating pool can then be disregarded. The boundary between the 'stem cell' and the differentiated pool is morphological. Errors in classifying cells in this region have little effect, however, on the estimated size of the differentiated pool because such doubtful cells constitute only a very small part of the differentiated pool. The actual time relationship between mitosis in the 'stem cell' pool and transition to the first differentiated compartment is not known, but fortunately there is information on the last mitosis of the proliferating pool. In this case it is very important that the relationship between mitosis and transformation is known because the numerous daughter cells from the last mitoses are, of course, non-proliferative. It has been concluded that the last mitosis in a differentiated cell line, at least within erythropoiesis, is nearly simultaneous with morphological transformation into the non-proliferating pool [62]. The pool labelling index is a function of the number of generations within the pool, the transit times of successive generations and the

duration of S phases. If the cytologic classification is strictly uniform throughout an investigation, the values of labelling indices of individual compartments are useful in indicating differences between different cases. By combining the labelling data from all supposedly similar cases more reliable values of compartment labelling indices are obtained.

When estimating the labelling index of a pool or of a compartment a possible uneven cell distribution at any stage of the method can be disregarded.

As with differential counting the statistical counting error must be considered. The relative variation due to this can be reduced only by increasing n_i , the total number of a special cell type, i , counted. Combining together compartments into pools gives an increased value of n_i . The n_i values obtained for the proliferating pools in the differential count of 1,000 cells were, however, still too small and additional cells were therefore counted. During the preliminary experiments, the lowest labelling index, i.e. p_i of about 0.10 was noted in the 'stem cell' pool of certain patients. As other methodological errors seemed to be relatively small, a relative statistical counting error of about 20 % was considered satisfactory for this p_i value. This necessitated a count of about 200 cells, and additional counts up to $n_i = 200$ were made in all proliferating pools. To reduce the relative error to 10 % when $p_i = 0.10$ would require a count of about 1,000 cells, which was a virtual impossibility for practical reasons.

Estimate of error in calculation of labelling index. This error was estimated by comparing the labelling indices of the three main proliferating pools obtained at repeated examinations of one bone marrow aspirate from each of eight healthy children. In every case the analyses were repeated twice on the same autoradiogram and made once on a second autoradiogram with a different film-date. By analyses of variance no significant differences were found between the values of the double analysis of the same autoradiogram and that of the second autoradiogram for any of the pools examined.

This test indicated a satisfactory reproducibility of the actual method. The relative variation due to the method error, including the statistical counting error, was roughly 8 % for the proliferating erythropoietic pool, 17 % for the granulocytopoietic pool, and 20 % for the 'stem cell' pool.

Errors of mean grain count. The preceding discussion about grain count and labelling index also applies to the mean grain count, but this is liable to certain additional errors.

The conditions for obtaining an optimal difference between 'true' labelling and background granulation were established in preliminary experiments. The average density of background grains was 0.2 grains per $100 \mu^2$. Table 2 shows that the frequencies of cells with 4 and 5 grains over their nuclei are very low. Such cells in fact constituted less than 2 % of all cells with one or more grains above their nuclei. Thus the 4 or 5 grain level

TABLE 2. *Percentage grain count distribution in proliferating bone marrow cells, pooled from differential and grain counts of 5 healthy children.*

Grain count	1	2	3	4	5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	>40
Percentage of total cells	23.4	5.9	1.3	0.9	0.9	5.1	6.0	7.3	6.2	4.5	5.0	2.4	31.1

can be regarded as the borderline between labelling and non-labelling. On the other hand, high grain counts are undesirable as the difficulties of accurate grain count become severe with more than 60-70 grains per cell. Grain counts from 6 to 40 were classified in groups of five and then as 'more than 40'. This last group was counted as '50' in calculating the mean grain count. This may have possibly levelled to some extent the differences between the mean grain counts of different cell types of the same series. The average grain count of the 'more than 40' class is probably higher for primitive than for more mature cells. When mean grain counts of the same pool were compared in different patients, this error was assumed to be of less

importance since the relative proportions of individual compartments within the pools and the labelling indices of the pools compared were about the same size. When the results were interpreted, mainly the mean grain counts of pools were taken into consideration.

Estimate of errors of mean grain count. This error was estimated in the same way as for the labelling index. The mean grain count of different pools in each of 8 healthy children was calculated twice on the same autoradiogram and once on an autoradiogram with a different film-date. Analysis of variance showed no significant difference between the mean grain counts obtained from the same or a different autoradiogram.

CHAPTER III

Healthy infants and children

Clinical material

The cellular composition and ability to incorporate ^3H -thymidine *in vitro* were studied in bone marrow specimens from 53 infants and children aged from seven hours to eight years. In seven additional cases the *in vitro* labelling experiments failed but their differential counts were included in the analysis of the total material.

The mothers of the infants investigated were healthy during pregnancy, and the infants were born in a maternity hospital at full term after normal labour. At the time of the investigation the infants appeared healthy and had normal blood values. All the neonatal cases were born in the same county hospital (Enköpings). The infants aged from one week to one year were all either ward-
ed for social reasons* in a paediatric clinic (University Hospital, Uppsala), or were looked after in a Home for Healthy Infants (Gillbergska children's home, Uppsala). The children over one year old were in hospital for different non-haematological reasons and had no wasting or other serious disease.

The largest variations with age in

both the bone marrow and peripheral blood occur during the first months of life [44, 47]. Special attention was therefore paid to the situation during this period and more especially to the first week of life. 31 infants under one month old were studied, 14 of them being less than one week old. 24 cases aged from 1–12 months and 5 cases, aged from 18 months to 8 years were also investigated. The relative fewness of children over one year old in the study was partly motivated by the conclusion of Glaser et al. [47] that there are no consistent changes in the bone marrow picture between 1–20 years of age. It was also difficult to collect healthy cases from this age group. The age distribution of the cases investigated is given in the *Appendix* and with the figures describing the results. In analysing the results, the cases were divided into the following age groups: '0–1 month', '1–12 months', and 'above 12 months'.

Results

Differential counts

Differential counts of 1,000 nucleated cells in bone marrow smears from each child are given in the *Appendix*.

* For example, children awaiting adoption.

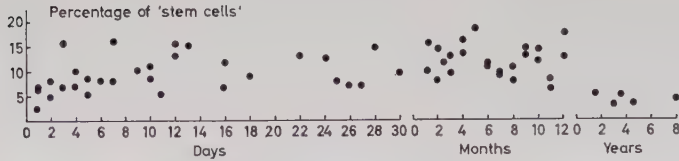


Fig. 5. The percentage of 'stem cells' in differential counts of 1,000 bone marrow cells in 60 healthy children of various ages.

In figures 5, 6, 8, and 9 the calculated percentage cell distributions are plotted against age. The figures show the erythropoietic and granulopoietic series, the lymphocytes, and the 'stem cells' (non-differentiated proliferative cells). In figures 6 and 8 which deal with the differentiated cell lines, the percentage of the proliferating pool is also plotted.

The results of the present study indicate certain characteristic bone marrow changes with increasing age. The variation due to the relative error of

differential cell counting and to individual biological variations means that only large changes can be regarded as significant.

'Stem cells' (Fig. 5). There were relatively small changes in the proportion of 'stem cells' during the first year (mean value $10.8\% \pm 3.8\%$). These changes are small compared to the relatively large changes in other bone marrow cells. The mean value of the five children more than one year old, however, was much lower ($4.2\% \pm 1.0\%$).

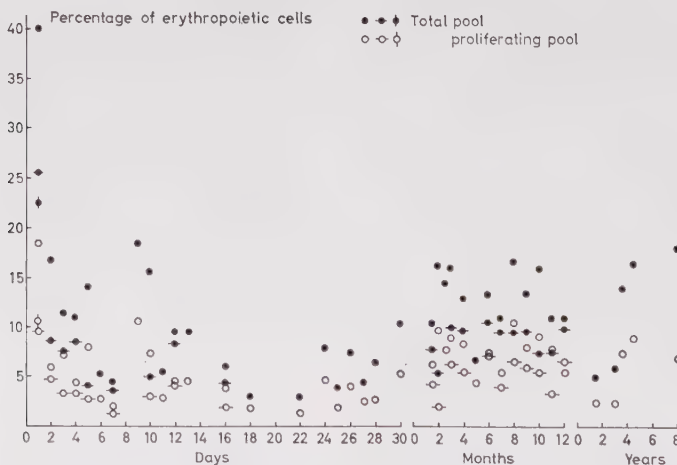


Fig. 6. The percentage of erythropoietic cells in differential counts of 1,000 bone marrow cells in 60 healthy children of various ages. The total number of cells in each case is indicated by a filled circle and the number of proliferating cells with an unfilled circle. Identical line symbols on two circles at the same age indicate that they are derived from the same differential count.

Erythropoietic series (Fig. 6). On the first day of life the bone marrow specimens contain on an average 30 % erythropoietic cells. This value falls rapidly to about 10 % during the first two-three days after which there is a further slow decline until the third week by which time it has decreased to about 5 %. During the next month there is a gradual rise to 10–15 %, a value which remains fairly constant during the first year of life. The results obtained in 5 children above this age showed about the same range.

The fraction of proliferating erythroblasts among the erythropoietic cells remains essentially constant except during the first week or two of life. There are reasons for believing that the ratio $Er-NP/P$ will increase during the first day or two after birth but due to insufficient material this has not been established experimentally.

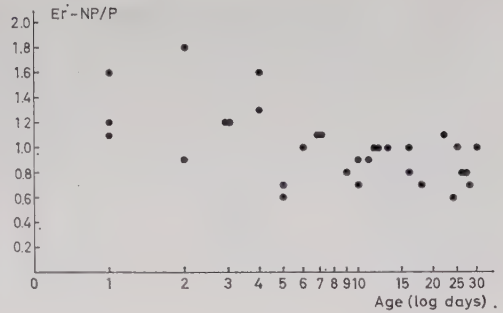


Fig. 7. The ratio between non-proliferating and proliferating erythroblasts ($Er NP/P$) in differential counts of 1,000 bone marrow cells in 31 children during the first month of life. The age in days is plotted on a logarithmic scale. There is a significant decline in the ratio over the first two or three weeks.

Subsequently there is a significant decline in the ratio over the next two or three weeks, the regression coefficient being significantly different from zero in the linear regression of the ratio $Er-NP/P$ on the logarithm of the age (Fig. 7). These matters are further discussed on p. 49.

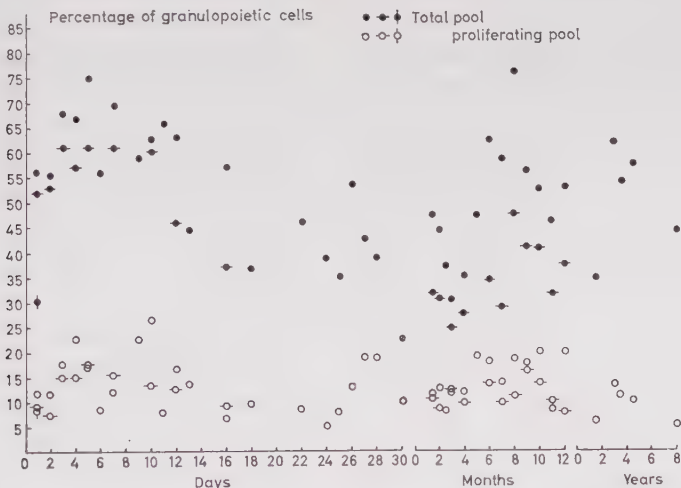


Fig. 8. The percentage of *granulopoietic cells* in differential counts of 1,000 bone marrow cells in 60 healthy children of various ages. The total number of cells in each case is indicated by a filled circle and the number of proliferating cells with an unfilled circle. Identical line symbols on two circles at the same age indicate that they are derived from the same differential count.

TABLE 3. *Proportional distribution of erythropoietic and granulopoietic cells in bone marrow differential counts of 60 healthy infants and children.*

Cell type	Erythropoietic cells				Granulopoietic cells		
	Er-1	Er-2	Er-3	Er-4	Gr-1	Gr-2	Gr-3
Number of cells relative to compartment 1	1	6	13	18	1	1.6	8.3
Ratio NP/P	0.9				3.2		

Granulopoietic series (Fig. 8). On the first day after birth about 45 % of the nucleated cells of the marrow belong to the granulopoietic series. During the next 2-3 days they rise to 60-65 % and then fall to about 40 % on the 12th-14th day. This level is then steady to the fifth or sixth month whereafter it again rises to about 50 % with large individual variations.

The percentage value of the proliferating pool is also given in figure 8. The figure indicates that variations in the size of the total granulocytic pool depend more upon changes of the non-proliferating than of the proliferating pool. This is mainly due to changes in size of the mature granulocyte pool (cf. *Appendix*); a large number of

mature granulocytes in the marrow are held as a reserve which undergoes considerable fluctuations. The ratio Gr-NP/P has therefore a wide range and its value is thus normally of limited usefulness. It showed no significant correlation with age, the mean value being 3.2 ± 1.6 . In certain pathological cases there were, however, significant deviations from this value.

The proportional distribution of individual cell compartments in the erythropoietic and granulopoietic series (Table 3) was obtained by pooling the values of all healthy cases in the present investigation. The size of each compartment is given as a ratio to the size of the first compartment in the series (Er-1 and Gr-1).

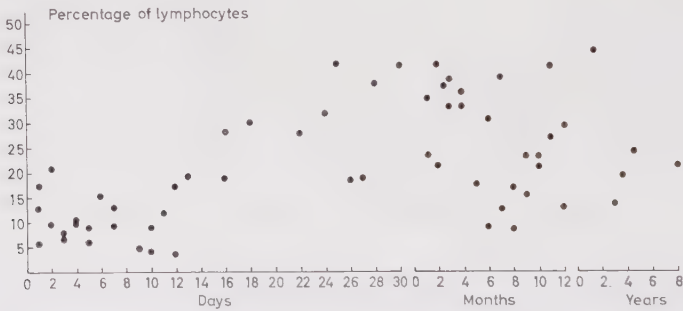


Fig. 9. The percentage of *lymphocytes* in differential counts of 1,000 bone marrow cells in 60 healthy children of various ages.

TABLE 4. *Percentage of eosinophil granulocytes in bone marrow differential counts.*

Age period (month)	0-1	1-12	>12	(Total)
Number of cases	31	24	5	60
	mean (S.E.)	mean (S.E.)	mean (S.E.)	mean (S.E.)
Immature cells	1.2 (0.1)	1.4 (0.1)	1.3 (0.4)	1.3 (0.1)
Mature cells	2.9 (0.2)	1.3 (0.2)	3.6 (1.4)	2.3 (0.2)
Total	4.1 (0.2)	2.7 (0.1)	4.9 (1.1)	3.6 (0.2)

Lymphocytes (Fig. 9). During the first 10-12 days of life there is a low lymphocyte value of about 10 %. This value increases markedly during the next fortnight up to about 35 %, after which there seems to be a decrease during the following months to about 20 % at the end of the first year. This value then subsequently remains fairly stable.

Cells occurring in small numbers. The following cells were sparse in the bone marrow. Changes in their numbers were therefore difficult to evaluate.

(a) *Eosinophil granulocytes* were present at all ages (see *Appendix*). In children below one month and above 12 months of age there were somewhat larger percentage values because of a smaller pool of mature eosinophil granulocytes between 1-12 months of age (Table 4).

(b) *Basophil granulocytes* were either lacking, as in most of the differential counts, or occurred only rarely (range 0.1-1.0 %). No relationship to age was found.

(c) *Monocytes* were seen in smears of all cases except four (mean 1.5 %, range 0-7.9 %). There appeared to be no relationship to age.

(d) *Plasma cells* were usually so few in number that they did not appear in the differential counts of 1,000 cells. In children under one month old no plasma cells were found in the differential counts (see *Appendix*).

(e) *Megakaryocytes* were occasionally present in some of the smears but were not included in the differential counts (cf. p. 15).

Disintegrated cells were discussed on page 18 (cf. *Appendix*). No relationship with age was found.

Labelling indices

The labelling indices of the non-differentiated proliferative cells, the 'stem cell' pool and of the proliferating pools within erythropoiesis and granulopoiesis, are plotted as functions of age in figures 10-12. The labelling indices noted in each child are given in the *Appendix*.

The labelling index of the 'stem cell' pool (Fig. 10) varied during the first month of life. The values in the first week were low and then increased until the fourth week after which they remained, on the average, essentially unchanged. The increase during the first month was significant ($P < 0.01$).

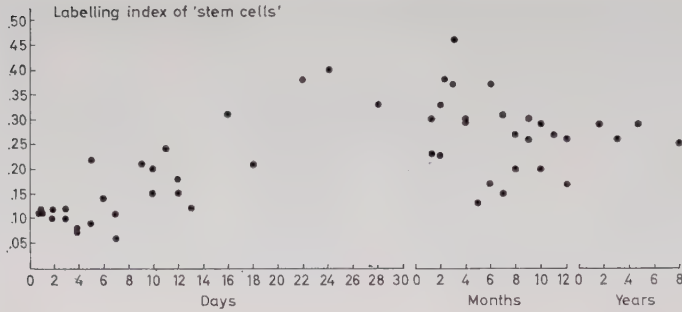


Fig. 10. The labelling index of 'stem cells' in the bone marrows of 53 healthy children of various ages after a one hour incubation of marrow in the presence of tritiated thymidine. The calculation is made from 200 cells in each case. There is a significant increase during the first month but subsequently no significant change.

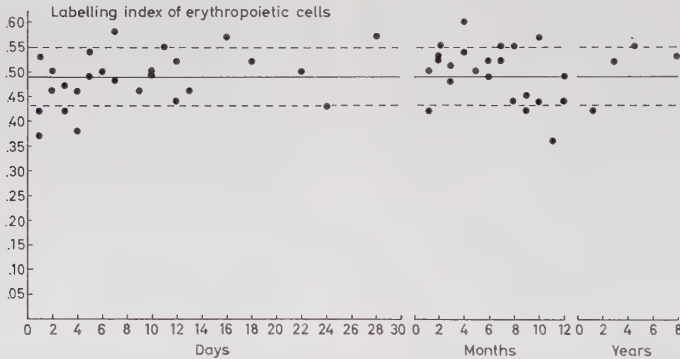


Fig. 11. The labelling index of the proliferating *erythropoietic pool* in the bone marrows of 53 healthy children of various ages after a one hour incubation of marrow in the presence of tritiated thymidine. The calculation is made from 200 cells in each case. The mean value (.49) is indicated by a continuous line and ± 1 standard deviation ($\pm .06$) by the dashed lines. There is no significant change with age.

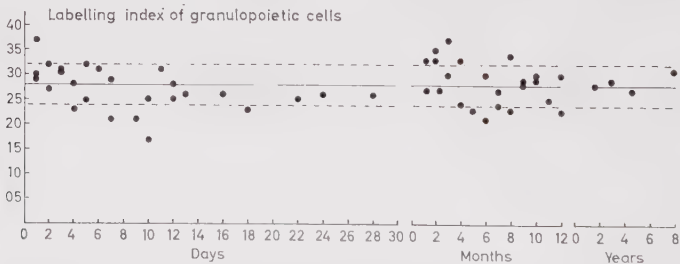


Fig. 12. The labelling index of the proliferating *granulopoietic pool* in the bone marrows of 53 healthy children of various ages after a one hour incubation of marrow in the presence of tritiated thymidine. The calculation is made from 200 cells in each case. The mean value (.28) is indicated by a continuous line and ± 1 standard deviation ($\pm .04$) by the dashed lines. There is no significant change with age.

TABLE 5. Mean labelling indices (L.I.) of proliferating erythropoietic and granulopoietic cells in bone marrow investigations of 53 healthy infants and children.

Cell type	Erythropoietic cells				Granulopoietic cells		
	Er-1	Er-2	Er-3	Er-P	Gr-1	Gr-2	Gr-P
L.I.	.52	.63	.42	.49	.32	.25	.28

Figures 11 and 12 show that there were no significant variations of the labelling indices of the *proliferating differentiated pools* with increasing age. The mean values were $.49 \pm .06$ in the erythropoietic pool and $.28 \pm .04$ in the granulopoietic pool, calculated on all 53 cases.

Labelling indices of the different *proliferating compartments* were obtained by pooling the labelling data of proliferating cells from all healthy cases. These values are given in Table 5.

Bone marrow *lymphocytes*, as defined above were not found labelled. Some of the immature cells within the eosinophilic and monocytic cell series were labelled but in too few numbers to allow any conclusions as to their significance.

Labelled *disintegrated cells* constituted about 1 % of the differential counts (range 0-3 %). The proportion of disintegrated cells labelled, i.e. the labelling index, was, on an average, .18 (range .05-.33) without any correlation to age. This means that only a small number of proliferative cells were disintegrated during the technical procedure. The labelling indices of the proliferating pools therefore would not be materially influenced.

Mean grain counts

The mean grain counts of the three proliferating pools studied are given in relation to age in Table 6. There was no significant difference between children of different ages for any proliferating pool.

Higher grain counts were observed in more immature cells, as is shown in Table 7. The mean grain counts of individual cell types within the differentiated proliferating pools are given as the mean values of all children studied.

TABLE 6. Mean grain counts of proliferating bone marrow pools at various ages.

Age	Number of cases	Proliferating pool		
		Er	Gr	St
week				
1	14	32	32	36
2	7	37	39	41
3	2	26	30	38
4	3	32	32	34
months				
2-3	7	25	32	32
4-6	5	23	31	36
7-9	6	23	31	32
10-12	5	31	35	40
> 12	4	26	31	34
Total material	53	30	34	38

TABLE 7. *Mean grain count of proliferative bone marrow cells within erythropoiesis and granulopoiesis. Values are the pooled means of 53 children.*

Cell type	Erythropoietic cells			Granulopoietic cells	
	Er-1	Er-2	Er-3	Gr-1	Gr-2
Mean grain count	44	36	25	37	32

Discussion

Comparison with earlier investigations

Differential count

The literature, up to 1950, concerning the cellular composition of the bone marrow in healthy infants and children was reviewed by Glaser et al. [47] in their study of 151 cases aged from one day to 20 years. Later Gairdner et al. [44] investigated 103 bone marrow specimens from 25 infants aged from a few minutes to three months. Sturgeon [109] studied 72 marrow aspirations from 50 normal infants aged from 5 hours to 11 years. The conclusions drawn from these studies are in good agreement as regards the general pattern of the changes occurring in bone marrow specimens with increasing age. When, however, detailed comparisons are made, great variations with respect to the percentage distributions of different cell types become apparent. These variations may be due to differences in cell nomenclature and classification and/or to different methods of bone marrow collection.

The results of the present study are in good agreement with the above cited investigations as concerns the changes in percentage distribution of the *erythropoietic* series, the *granulo-*

poietic series and *lymphocytes*. The actual percentage values found in the erythropoietic series were, however, somewhat lower.

Both Glaser et al. and Gairdner et al. found good evidence of a marked decrease in the total number of nucleated bone marrow cells during the first week of life; this was due mainly to a fall in the number of nucleated erythroid cells. The percentage increase of the granulopoietic series during the same period is only relative, since according to Gairdner et al. there is probably a decrease in the absolute number of these cells. The obvious increase in lymphocyte percentage during the second and third weeks corresponds to an increase in absolute numbers.

A 'new' cell group, 'stem cells' consisting of non-differentiated, proliferative cells is introduced in the present study. No direct comparison with other investigations can therefore be made. 'Stem cells' were found in significant numbers in all the smears investigated. This cell group, however, includes not only the 'myeloblast' and 'lymphoblast' of current nomenclature, but also other cell types, because the proportion of the 'stem cells' was larger than that found by other investigators for 'myeloblasts' and 'lymphoblasts' combined. The 'stem

cell' pool would be apparently increased if early stages of differentiated cells were included. This is not probable as the cytologic criteria of the proerythroblast, the promyelocyte, the immature monocyte, etc., appear to be the same in this study and in most other investigations. There are some technical aspects of the present study that may explain the relatively large size of the 'stem cell' pool which was found.

(1) The autoradiographic studies aided in the classification of proliferative and non-proliferative cells. According to the criteria already given, 'stem cells' are proliferative cells and 'lymphocytes' are non-proliferative. In all bone marrow studies previously reported the cell group 'lymphocytes' or 'lymphoid cells', etc., seems to be rather heterogeneous and no regard has been taken as to whether or not these cells can proliferate. In the system used here proliferative 'lymphoid cells' are classified as 'stem cells' and will therefore contribute to a certain increase of the size of the 'stem cell' pool.

(2) Disintegrated or 'smear' cells are not included in the differential counts in most bone marrow studies, although they have often been reported to occur in high and variable numbers (cf. p. 18). In the present study, the presence of 'disintegrated cells' was smaller and less variable. Autoradiography revealed, however, that on an average about 18% of these disintegrated cells were labelled. This figure, of course, does not represent all the proliferative cells that were disintegrated since some of them will not have been

in the DNA synthesizing phase. Primitive cells are more disposed to mechanical distortion than mature cells and it seems therefore likely that the larger the frequency of disintegrated cells, the larger the 'loss' of undifferentiated, proliferative cells. The low frequency of disintegrated cells in this study will thus probably contribute to a larger relative size of the 'stem cell' pool compared to some other studies where there was a higher incidence of disintegrated cells.

Most of the children studied here were under one year of age, i.e. in a period of very intense growth. Since the proliferation rate of the 'stem cell' pool remains essentially constant after the first two or three weeks, a relatively larger 'stem cell' pool may be expected in these infants than in older children and adults in view of the demands of rapid growth.

In this study the *ratio between the number of non-proliferating and proliferating cells* within the differentiated pools was used for interpretation of the cellular kinetics. These ratios exhibit large variations in the bone marrow investigations quoted (Table 8).

The results presented here cannot be compared with those of Sturgeon [109] who classified polychromatic and orthochromatic erythroblasts together in one group and myelocytes and metamyelocytes together in another group, thus making it impossible to determine the ratio between proliferating and non-proliferating cells. The results of Gairdner et al. show large variations which are probably

TABLE 8. *Ratio between non-proliferating and proliferating pool (NP/P) in some bone marrow investigations.*

Reference	Material		Er-NP/P	Gr-NP/P
	number of cases	age range (years)		
Gairdner et al. [44]	25	0—3/12	1.0—3.0	3.7—6.5
Present investigation	60	0—8	0.9 ^a	3.2 ^a
Glaser et al. [47]	151	0—20	0.1—0.4	1.5—1.9
Osgood and Seaman [83]	473	adult	—	3.1 ^a
Killmann et al. [62]	6	adult	0.83 ^b	1.84 ^b

^apooled from total material^bmean value

due to their differential counts being based on 200 cells only. The very low erythropoietic ratio (Er-NP/P) reported by Glaser et al. must imply wide cytologic criteria for their classification of the polychromatic erythroblasts as these cells comprised no less than 80–90 % of all erythroblasts. In Table 8 ratios from two additional studies are given. Osgood and Seaman [83] critically reviewed data of bone marrow differential counts from healthy individuals. From the pooled data of differential counts from 473 adults only the granulopoietic ratio (Gr-NP/P) could be calculated because of the very variable criteria for classification of nucleated erythrocytes. Killmann et al. [63] reported the mean bone marrow differential counts of six healthy adults; this study is of special interest as they used ³H-thymidine labelling. Their mean value for the granulopoietic ratio of 1.84 is however considerably lower than the value of 4.0 that they obtained in another study on a single patient [31]. Comparisons between the results given by different investigators are thus not of great

value because of the large variations and other factors discussed above.

Neither is it possible to make strict comparisons between the results of different investigators as to the proportional distribution of individual cell stages. The values found in the present study are given in Table 3 and will be considered later in a comparison between healthy individuals and those with haematological diseases and in the interpretation of erythropoiesis in the neonatal period.

Labelling indices

No study has been published previously concerning *in vitro*-labelling with ³H-thymidine of bone marrow specimens from infants and children. The results of the present study can be compared therefore only with data from adult bone marrow. In some of these investigations, labelling data, but not the percentage distributions of the individual cell types, are given and a non-pathological distribution has to be assumed. Differences in cytologic characterization and technique make comparisons even more uncertain.

TABLE 9. *Labelling indices (L.I.) of differentiated proliferating pools in some bone marrow investigations.*

Reference	Number of cases	Er—L.I.	Gr—L.I.
Schmid et al. [97]	12	.40	.35
Mauri [81]	5	.50—.55	—
Lajtha [66]	not given	.47	.36
Cronkite et al. [15, 31]	not given	.50—.58	.25
Present investigation	53	.49±.06 ^a	.28±.04 ^a

^aThese figures are means $\pm$ 1 standard deviation. This notation is used in subsequent tables

Erythropoiesis and granulopoiesis.

The present study confirmed the general finding in the studies cited in Table 9, that the erythropoietic and granulopoietic cell series were labelled as far as the polychromatic erythroblast and the myelocyte, respectively. Table 9 also gives labelling indices of the differentiated proliferating pools; the indices obtained from the references cited were calculated from their data. Both the erythropoietic and the granulopoietic indices obtained in the present investigation appear to fall within the range given in the studies cited.

'Stem cells'. In the data of the studies cited above there is no cell defined that corresponds to the 'stem cells' of the present study. Bond et al. [15] noted 'a group of unidentifiable cells' that was occasionally very heavily labelled. They regarded these cells as 'a primitive progenitor pool'. 'Small mature lymphocytes' were not found labelled. Lajtha [66] described two types of 'lymphocytes' in the bone marrow; the great majority, i.e. 94 % of all 'lymphocytes' were small and were never found labelled, the remaining

6 % were large and had a labelling index of 0.30. His labelling data are, however, not strictly comparable with those of the present study.

Mean grain count

Mean grain counts of proliferating pools cannot be calculated from the data published in the papers cited above. Such a comparison would, in any case, probably be of restricted interest since the different techniques used by different investigators may well influence the mean grain counts. In general, it has been noted that the grain count is higher the more immature the cell, as was also the case in the present investigation (cf. Table 7). This difference in labelling between cells of varying maturity is consistent with the hypothesis that more immature cells have a faster DNA synthesis rate [66] but the larger nuclear size also contributes to a higher mean grain count in these cells.

In the present study, the mean grain count was used mainly for comparisons between children of different ages and between normal and pathological states.

The problem of the stem cell

On the whole, data about the 'stem cells' of human bone marrow are sparse in the current literature. On the other hand, there is a wealth of published material on the functional and cytologic characteristics of a bone marrow 'stem cell' in other animal species. Both *in vitro* and *in vivo* labelling with ^3H -thymidine has been frequently used in these investigations. The data indicate that a mononuclear cell with a lymphocyte-like appearance has 'stem cell' properties [33, 51, 111]. While small lymphocytes do not incorporate ^3H -thymidine in flash labelling or in short-term incubation, 'medium' and 'large' lymphocytes or 'lymphoid cells' do become labelled [52]. According to Craddock [26] the lack of uptake of ^3H -thymidine by the 'small lymphocytes' in the bone marrow may be explained as due to their larger intracellular thymidine pools or to reutilization of DNA. Harris et al. [51] described a proliferating mononuclear cell in guinea-pig bone marrow as having a characteristic 'transitional' morphology with the high nuclear-cytoplasmic ratio, typical of a lymphocyte, but with fine nuclear chromatin and basophilic cytoplasm, i.e. features of blast cells. This cell type appeared as a 'stem cell' in their irradiation experiments. Lewis and Trobaugh Jr [73] have given convincing evidence that, in mice at least, the bone marrow 'stem cell' is pluripotent. The definite identity of the 'stem cell' is still, however, unknown.

The kinetics of a bone marrow 'stem cell'-population has been the

object of much speculation and investigation. There are several theories regarding the transition from 'stem cell' to differentiated cell. With the aid of modern techniques, theoretical models of 'stem cell' kinetics have been tested experimentally. Osgood [82] and Lajtha et al. [68] have described and extensively discussed kinetic models. According to Lajtha's theory, the 'stem cell' pool is self-perpetuating and differentiative actions occur when the 'stem cells' are in the ' G_0 ' phase, i.e. the phase that precedes the generative cell cycle (G_1 -S- G_2 -M phases). Support for this theory has come from recent experiments with erythropoietin stimulation and neutralization of the differentiative activity with immune serum [98].

In Fig. 13 the development from 'stem cell' to mature blood cell is described schematically. The height (y-axis) of the areas of the cell types approximates the relative amount of a special cell stage, i.e. cell flow, and the length (x-axis) the relative transit time of each type. The stepwise increase of the 'cell pool areas' indicates the doubling of cell flow by mitoses within the proliferating pool; the cell flow through the non-proliferative compartments is unchanged. The following assumptions are made: the 'stem cell' pool is self-perpetuating and maintains a constant size; the differentiated pool consists of three generations, the last mitosis being heteromorphogenic; the duration of the S phase is constant [11]. The transition from marrow to blood is indicated by a vertical dashed line.

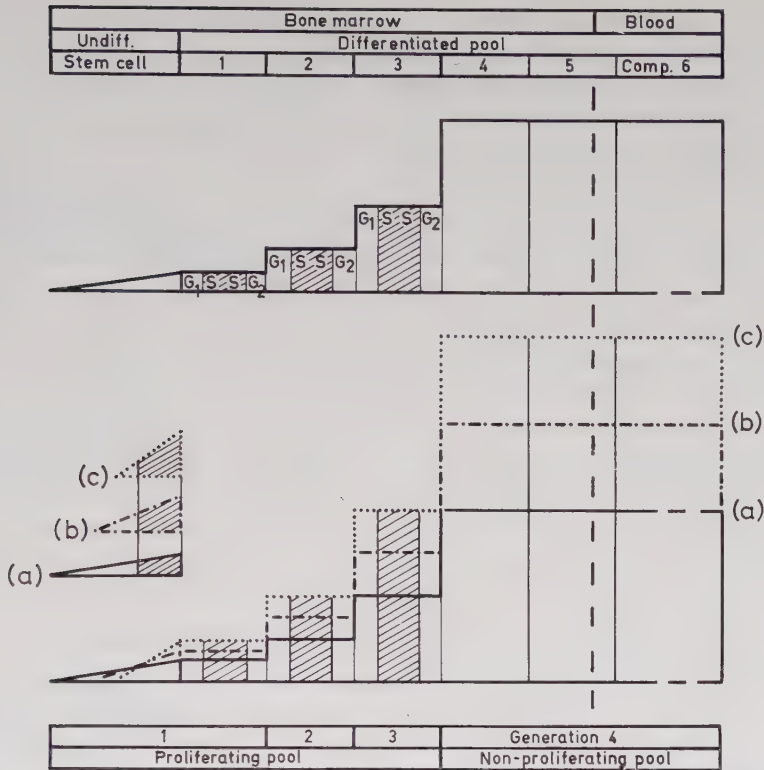


Fig. 13. A schematic outline of the possible effect of changed proliferative activity within a self-perpetuating 'stem cell' pool on the inflow into and outflow from a differentiated pool. The height on the ordinate of the areas of the cell types approximates the relative amount of that special cell type, i.e. cell flow, and the length on the abscissa the relative transit time of each type. Three alternative schemes (a, b, and c) are shown with the proliferative activity of a 'stem cell' pool, of constant size, lowest in (a) and highest in (c). The upper diagram which shows the generation phases G_1 , S and G_2 is identical to (a). The 'stem cell' pool is represented in each scheme by a triangle of equal area to indicate its constant size. The cell flow through the differentiated pool is duplicated stepwise by mitoses. Increase of 'stem cell' proliferation results in a proportional increase in outflow from the 'stem cell' pool. The different proliferation activities of the 'stem cell' pool in the three alternative schemes are indicated by the height and steeper slope of the triangle. The size ('area') of the differentiated pool and the outflow from it ('height') are greater in (b) and (c) because of the greater inflow into this pool. The proportional distribution and the labelling index remain unchanged, however, because the number of generations and the duration of the S phases are not altered and the terminal division in the pool is heteromorphogenic. See text for further comment.

In order to maintain its size the 'stem cell' pool must change its cellular proliferation in direct proportion to the outflow of cells into the differentiated pools. The proportion of 'stem cells' actually proliferating is a function of

the proportion of 'stem cells' in S phase, i.e. the labelling index.

A change of the inflow into the differentiated pool with an unchanged number of generations will markedly change the outflow from the differen-

tiated pool, i.e. the production of mature cells. In Fig. 13 three alternatives of inflow into the differentiated pool are given, resulting in different outflows from the pool but keeping its relative size and labelling index unchanged. A word of explanation regarding the representation of the 'stem cell' pool is necessary. For this pool the term transit time is meaningless since all cells supposedly originate here. When it is constant in size, it is represented in all the figures by a triangle of equal area. The 'stem cell' pool in alternative (a) has the smallest outflow (height, 'y') and therefore the lowest proliferation rate; this is represented by the least steep slope. Thus, (b) and (c) with successively increased outflows ('y'-values) have steeper and steeper slopes. Hence, to maintain the area constant, the base ('x') must vary in length but this does not imply an increased generation time in this pool.

As discussed it appears justifiable to classify the undifferentiated, proliferative cells of the bone marrow into a single group. Morphologically this cell group is heterogeneous, and its cytologic borderlines with the most primitive cells of the differentiated cell lines and with bone marrow lymphocytes are by no means clear cut. The cytologic description of the mononuclear, lymphocyte-like cell that appeared to have 'stem cell' properties in different animal experiments, has much in common with the description of the 'stem cell' given in this study. In the following discussion some evidence will be presented that points to certain

functional properties of the 'stem cell' pool.

Kinetics of the differentiated cell lines

The kinetics of *erythropoiesis* is assumed to follow a regular pattern (cf. Fig. 1, p. 7) under normal conditions [16]. There are many views about the regulation of cellular proliferation. According to Stohlmann [107], proliferation within the erythron is probably controlled by the haemoglobin content of the cell, and normally the haemoglobinization pattern is rather constant. Marked variations in the number of generations within the proliferating pool are unlikely, at least under normal conditions.

Within granulopoiesis there is much divergence of opinion about the pattern and regulation of proliferation. According to the 'classic' scheme [64] there is a regular schedule of maturation and division in the consecutive compartments. Boggs et al. [14] concluded that the myelocyte pool was to some degree self-perpetuating. Patt and Maloney [84] suggested that granulocyte production might be 'death-controlled' on the myelocyte level, i.e. adjusted by variations in the mortality of newly formed cells, analogous to 'ineffective' erythropoiesis (see below).

Apart from different views about the proliferation pattern, there are different morphological criteria of classification and incomplete knowledge of the interrelationship between generation changes and cytologic boundaries.

Consequently, the tentative generation times for erythrocyte and granulocyte precursors given by different investigators [89, 90] vary considerably.

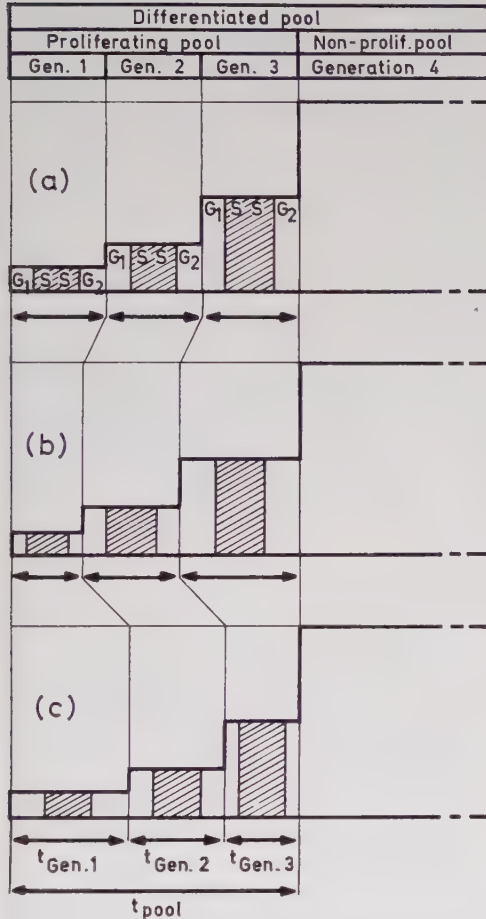


Fig. 14. A differentiated pool. A schematic outline of the effect on the proportional distribution and labelling index, of different generation times ($t_{\text{Gen.}}$) with the pool transit time (t_{pool}) unchanged. The duration of consecutive generations is the same in alternative (a), increases successively in (b), and decreases in (c). The proportional increase of cells in consecutive generations is most marked in (b) and least in (c) while the pool labelling index is highest in (c) and lowest in (b). The cell flow through the pool is, however, the same in all alternatives. Co-ordinates as in Fig. 13; see text for further comment.

The labelling index of a differentiated pool is the ratio between the estimated number of pool cells in S phase and all pool cells. Proportionally equal changes in the duration of S phases and all generation times will leave the labelling index unchanged. Such changes are not probable, however, as the DNA synthesis phase in any bone marrow cell generation appears to have a fixed duration of 7–8 hours [11, 21]. Changes in generation times would therefore generally be reflected in changes of the pool labelling index. The duration of consecutive generations in the pool, however, may not be the same and this will have consequences for the interpretation of the labelling indices. This is illustrated in Fig. 14 which schematically shows a differentiated pool with a constant pool transit time and various alternatives for generation times in the proliferating pool. In Fig. 14 (a) all three generations have the same duration, while there is in (b) a successive increase and in (c) a successive decrease of the duration from the first to the third generation. Of the three alternatives, (c) will apparently have the highest and (b) the lowest labelling index. The cell flow through the pool is the same in all three alternatives, however.

Changes of the outflow from the proliferating pool will in general be induced by changes in the inflow into the pool and/or in the number of mitoses within the pool. Changes in inflow alone or in the number of generations, if these are of equal duration, will not induce changes in pool labelling index.

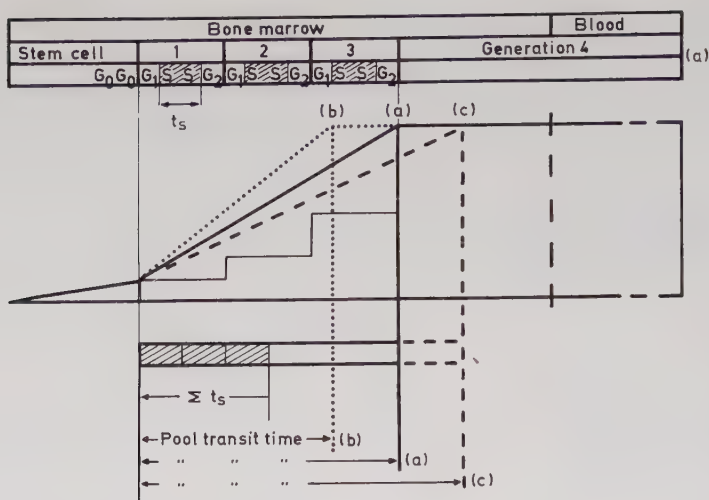


Fig. 15. A differentiated pool. A schematic outline of the effect on the pool labelling index of different pool transit times when consecutive generations are of equal duration. It is assumed that the number of generations is not changed and that the duration of the S phases (t_s) is constant. Thus, while the total duration of the three S phases (Σt_s) is constant, the duration of a single generation increases from (b) to (a) to (c). The pool labelling index is thus the ratio of Σt_s to the pool transit time. Co-ordinates as in Fig. 13; see text for further comment.

A change in the number of generations, if these are of unequal duration, will affect, however, the pool labelling index.

For the case of successive generations of equal duration, the pool labelling index will be a linear function of the pool transit times as is illustrated in Fig. 15. If the number of generations were known and if the S phase was of a constant duration, the pool transit times could be calculated.

Lajtha [69] and Alpen [3] gave evidence that some erythrocytes are derived from pro-erythroblasts which had matured without dividing, so called, 'skipped' divisions. Such a mechanism will reduce the outflow from

the differentiated pool and, if significant in extent, will affect the labelling index and proportional distribution, the magnitude of the effect being also dependent on the pool transit time of such cells. Irregular morphology of such maturing, non-dividing cells should also be expected.

The outflow can be reduced also by cell death within the pool, i.e. 'ineffective' haematopoiesis. Such a mechanism is probably of pathogenetic importance in certain haematological diseases, as will be discussed later. In this case, to interpret changes in proportional distribution and labelling indices, consideration must be taken of many factors: degree of cell death, place of cell death in pool and in gene-

ration phase, interrelationships between successive generation times, and possibly changed duration of generation phase. It has been postulated that a certain degree of 'ineffective' erythropoiesis occurs under normal conditions [106] but its magnitude and mechanism are not known.

The differentiated bone marrow cells leave the proliferating pool and move through the non-proliferating pool in a sequential fashion by the process of maturation and then enter the blood. Within granulopoiesis, at least, there is a large pool of mature cells in the marrow which serves as a reserve from which cells can be released upon demand [28, 42]. It is possible that bone marrow reticulocytes also provide a corresponding although small erythrocyte reserve [118].

Bone marrow changes with increasing age

In the present study, the results of the differential counts indicated a relatively small variation in the percentage distribution of '*stem cells*' during the first year of life. The labelling index of the 'stem cell' pool was low during the first week and then gradually rose during the second and third weeks to a level which remained more or less constant thereafter (Fig. 10). The low index during the first week of life will be discussed in the section on haematopoiesis in the newborn period (see below).

The 'stem cell' index fluctuated more than the indices of the differentiated cell lines. This may have depended

both upon larger methodological errors in the calculation of the labelling index of the 'stem cell' pool and also on wider physiological variations in proliferation within the pool. The latter will lead to corresponding variations in outflow to the differentiated cell lines.

The first year of life is characterized by an intense physical growth. If a supposed 'stem cell' pool were responsible for the absolute increase of the differentiated pools without changing its own absolute size, it would necessitate a marked increase in proliferation and a decrease in its relative size. On the other hand, a moderate increase of the 'stem cell' pool would keep both its relative size and proliferation constant. The latter alternative accords with the findings of this study, i.e. moderate changes in percentage distribution and of labelling index after the neonatal period. Whether its absolute increase is due to formation of new 'stem cells' inside the marrow or influx from an extramedullary source is at present unknown.

The lower percentage values found in the children over one year of age may be just chance low values due to the small number of cases, but would fit in with the concept of a 'stem cell' pool stabilized by negative feed back. Some transformation of defined 'stem cells' to 'resting' reticulum cells ('fat cells?') is also possible [91].

The changes in *erythropoiesis* and *granulopoiesis* in infants and children with increasing age have been given above. During the first period of life the changes in the bone marrow findings were so marked that the newborn

period will be treated separately (see below).

The ratio between non-proliferating and proliferating erythroblasts showed significant changes during the first month of life but thereafter was relatively constant. There is little point, as discussed on p. 32, in calculating the ratio for the granulopoietic series for comparison between single cases.

The labelling indices of the erythropoietic and granulopoietic proliferating pools exhibited no significant change with age. According to the discussion above, these findings indicate relative constant, though not necessarily equal durations of the different generations within the pools. They confirm the assumption of a rather fixed maturation and proliferation schedule of the differentiated, proliferating pools.

The findings of the present study do not rule out the possibilities of 'skipped divisions' and 'ineffective haematopoiesis'. They do indicate, however, that under normal conditions such mechanisms affect bone marrow cell production to a rather constant (and probably small) extent.

Within erythropoiesis there are probably 4 generations within the proliferating pool [62]. Assuming fairly equal generation times and an S phase of 7.5 hours, a mean labelling index of .49 as obtained in this study would indicate a pool transit time of 61 hours. This estimate accords well with values given by many other investigators [89].

In this study the promyelocyte was

assumed to be the most immature granulopoietic cell. The mean labelling index of the proliferating pool was found to be .28. Using the 'classic' proliferation scheme with its assumption of 4 generations with fairly equal generation time [64], the pool transit time calculated from the present data is 108 hours. Other investigators have found a very wide range of the pool transit time, but mean values around 100 hours are common [22, 63, 90].

During the third and fourth weeks of life there was an increase, both relative and absolute, of *lymphocytes* in the bone marrow. The labelling index of the 'stem cell' pool rose concurrently though the size of this pool remained relatively unchanged. At later periods of life, however, changes in lymphocytes and the 'stem cell' labelling index do not run in parallel. Also there are increases in the erythropoietic and granulopoietic series during the third and fourth weeks. For these reasons the lymphocyte increase may not be related directly to rise in the 'stem cell' labelling index, and the increased lymphocytes may have come from other sources.

Neonatal period. The fall in haemoglobin and red cell counts in peripheral blood in the first weeks of life is generally accepted as mainly due to a decrease in bone marrow production of erythrocytes and also to a certain extent to the shorter survival time of erythrocytes in the neonatal period [45, 118]. The primary cause of the decreased red cell production is the adjustment to an environment with a richer oxygen supply [44, 49]. Hy-

poxia stimulates the production of erythropoietin (erythropoietic stimulating factor, ESF), which regulates erythropoiesis mainly by inducing the differentiation of 'stem cells' to erythroid cells [39]. Further development of nucleated red cells may also be subject to hormonal influences [17], possibly by a factor closely associated with ESF [74]. Halvorsen [49] and Mann et al. [78] found increased levels of ESF in cord plasma from normal neonates but no ESF was detectable in plasma from normal infants later on during the first weeks of life. They concluded that ESF had a regulatory action of erythropoiesis also in the neonatal period. The production of ESF is probably sharply reduced within a few hours after birth as judged also from the reticulocyte levels in infants during the first week [100] and reticulocyte response in experimental conditions [49].

The data obtained in this study provide additional evidence for some of the concepts on erythropoiesis in the neonatal period, which are discussed above. Figures 16 A-C illustrate a tentative model of erythropoiesis from the immediate prenatal period to approximately 6 days after birth.

The youngest infant in the present study was 7 hours old, and therefore the pictures proposed for both the immediate pre- and postnatal periods are rather speculative. Because of the large erythroid mass and high level of cord blood ESF in neonates the immediate prenatal picture has been conceived as in Fig. 16 A. Here it is assumed that there is a very active proliferation, i.e.

a high labelling index in the 'stem cell' pool, stimulated by a high level of ESF (indicated with a broad arrow) and resulting in a large mass of erythroid cells.

Figures 16 B and C treat the situation from the first to the sixth day. Here some relevant experimental data have been used in the model. The rapid fall in blood ESF just after birth (thin arrow in Fig. 16 B) is assumed to reflect a corresponding reduction of ESF activity in the marrow and hence a decrease in the differentiation of cells into the erythroid series. To maintain its size constant the 'stem cell' pool must reduce its proliferative activity, i.e. the labelling index decreases, as illustrated by the lessened slope of the 'stem cell' pool in Fig. 16 B.

Reduced proliferation in the 'stem cell' pool results in a lower influx of cells into the erythroid series which becomes progressively reduced in size during the following days. The pattern of proliferation and maturation within Er-P is, however, unchanged throughout the 6-day period, i.e. unchanged number of generations and duration of generation times. This statement is motivated by the observation that the labelling index of the erythropoietic proliferating pool was essentially constant through the whole material, i.e. 7 hours old to eight years.

Fig. 16 C describes the situation at different times after birth. After 2 days the size of Er-P is reduced; this is shown in the figure by the removal of the areas (2)' from the first generation and (2)'' from the second generation of the pool. The extent to which the

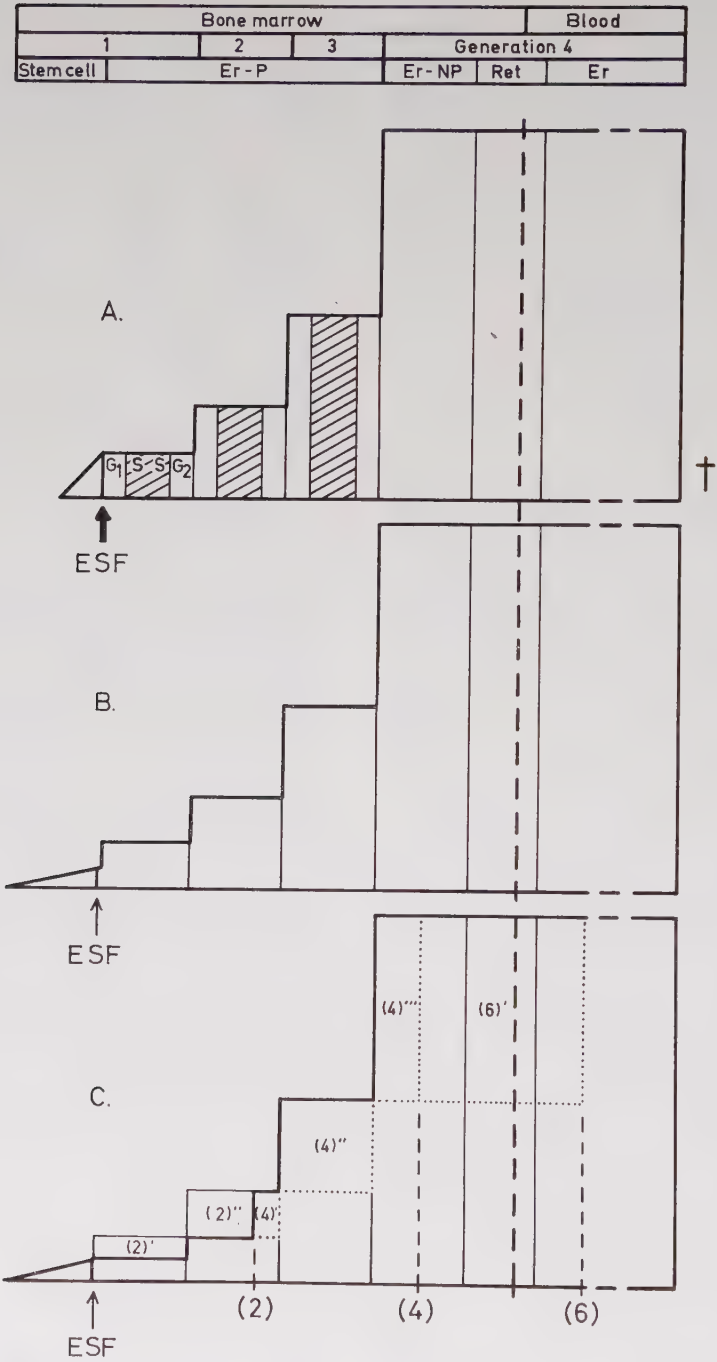


Fig. 16.

reduction 'tide' has moved through Er-P is indicated on the x-axis by the dashed line marked (2), and at this time the non-proliferating pool is as yet unaffected. On the fourth day, (4), the reduction 'tide' (removal of areas (4)', (4)'', and (4)''', delimited by dotted lines) has reached the non-proliferating pool. On the 6th day, (6), further reduction in this pool occurs with progress of the reduction 'tide' (removal of area (6)', delimited by dotted lines), the influx into the peripheral blood now being affected. With the passing of the reduction 'tide' out of the bone marrow all effects of the high foetal level of ESF have disappeared.

The points of time, indicated in Fig. 16 C, were suited to the schematic model in order to simplify the description of the effect of the 'tide'. Seip [100] noted a rapid drop in blood reticulocyte level around the fourth day after birth; the line for day (4) would therefore roughly coincide with the

line marking release of reticulocytes into blood.

According to the model, the reduced influx into the proliferating pool (Er-P) causes an initial increase of the ratio Er-NP/P since at first only Er-P and not Er-NP is reduced. When the non-proliferative pool also becomes reduced then the ratio declines again. As mentioned, the youngest child in the present investigation was 7 hours old and thus evidence as to the postulate of an immediate neonatal rise in the ratio is lacking. Differential counts, however, showed that in the children under one month old there was a significant linear regression between the NP/P ratio and the logarithm of age (Fig. 7, p. 31).

In contrast to the erythroid series the 'stem cell' pool exhibited a depressed labelling index neonatally. It seems reasonable to suppose that this indicates both a depressed proliferative activity and a decreased output into the differentiated lines. This could be

Fig. 16. (opposite page) A schematic outline of neonatal erythropoiesis. Diagram A represents the immediate prenatal picture with a large erythroid mass and a high cord blood level of erythropoietin (ESF) indicated with a broad arrow. A very active proliferation in the 'stem cell' pool is assumed in order to give the corresponding large inflow into the erythropoietic pool. Diagram B represents the situation a few hours after birth. The much lower ESF blood level is indicated with a thin arrow. The low labelling index of the neonatal 'stem cell' pool, found in this study, is interpreted as indicating a low proliferative activity and hence a decrease in the transformation of 'stem cells' to differentiated cells. This is symbolized by the lessened slope and height in the triangle that corresponds to the 'stem cell' pool; the size of this pool is constant. The erythropoietic pool is the same size as in A. Diagram C illustrates that the effect of the reduced inflow into the erythropoietic pool is like a 'tide' mowing through the pool which leaves a decreased erythropoietic mass in its wake as it progresses through the pool. The positions of the front of the reduction 'tide' at approximately 2, 4 and 6 days are indicated by the figures below the diagram. The proliferative activity within the erythropoietic pool (Er-P) is unchanged (constant labelling index). At day (2), Er-P is reduced by the areas (2)' and (2)'' and at day (4) also by areas (4)' and (4)''. Also at day (4) the non-proliferating pool (Er-NP) is affected by removal of area (4)''' and at day (6) a further reduction in this pool has occurred by removal of area (6)'. Thus, the reduced influx into Er-P causes an initial increase of the ratio Er-NP/P since at first only Er-P and not Er-NP is reduced. When Er-NP also becomes reduced the ratio declines. Co-ordinates as in Fig. 13; see text for further comment.

manifest as the reduction 'tide', induced by withdrawal of ESF and passing through the erythroid pool. These findings also indicate that the undifferentiated, proliferative cells of the bone marrow, designed as 'stem cells' in this study, seem to function as would be expected by a self-perpetuating 'stem cell' pool.

A very rapid decrease in haemoglobin synthesis and erythrocyte formation after birth is indicated by the analyses of radioiron data [45] and reticulocyte level [100] during the first ten days of life. The findings of the present study are in good agreement with such a statement. They further indicate that the immediate cause of this decreased production is a rapid decrease after birth in the inflow into the erythroid pool. The decrease of the Er-NP/P ratio and the constant labelling index were interpreted as evidence of the relatively constant proliferation and maturation schedule of the erythron in spite of a reduced flow.

The pattern of neonatal *granulopoiesis* has some features in common with erythropoiesis, although far less data are available. The full-term neonate has usually a polynuclear leucocytosis which increases during the first day and then subsides during the following three to five days. This may be provoked by transient factors such as foetal anoxia, stress during birth, local placental necrosis and possibly maternal stimuli, giving an effect analogous to that induced by the high

prenatal ESF level. Experimental data for the existence of such factors in human serum have been reported [104]. The total production of granulocytes in the marrow is reduced in the neonatal period [44] although relatively far less than the simultaneous reduction in erythropoiesis. These factors influencing granulopoiesis may also affect the 'stem cell' pool, through a mechanism analogous to the ESF effect, but the results of this study do not permit any conclusions in this respect.

The results of the present study indicate that there are no very great alterations in the generation numbers and transit times within the differentiated cell lines with increasing age in healthy children. Once a 'stem cell' is transformed into a differentiated cell, it seems to follow a rather fixed scheme as regards both proliferation and maturation. The production of cells, therefore, depends mainly upon the influx from the 'stem cell' pool into the differentiated cell lines. Assuming that the 'stem cell' pool is self-perpetuating and constant in size, a changed output necessitates a corresponding change in the proliferative activity within the pool. The ^3H -thymidine labelling index is a parameter describing this proliferative activity. As has been shown, it was essentially constant except for a significant depression in the neonatal period.

CHAPTER IV

Infants and children with haematological disease

Different haematological diseases were selected for the study of anomalous bone marrow cell proliferation. These diseases were: Haemolytic di-

sease of the newborn, Congenital hypoplastic anaemia, Infantile genetic agranulocytosis, and Aplastic anaemia.

A. HAEMOLYTIC DISEASE OF THE NEWBORN

Clinical material

Five neonates with mild haemolytic disease were studied. The criteria for selection were: (1) mature neonate with history of uncomplicated delivery;

(2) signs of haemolytic disease due to incompatibility within Rh-groups between mother and child and where exchange transfusion has to be seriously considered. The clinical and laboratory data are given in Table 10. Bone

TABLE 10. *Clinical and laboratory data in infants with Haemolytic disease of the newborn.*

Case No.	Siblings		Maternal titre		Infant at birth			Cord blood			Age at bone marrow aspiration (hours)	Bilirubin before exchange transfusion (mg %)
	No.	Complication due to Rh-incompatibility	Coombs' indirect test	Papain test	Weight (g)	Enlargement of Liver	Spleen	Coombs' test	Hb (g%)	Bilirubin (mg %)		
1	0	—	1:80	320	2,850	+	+	+	14.2	3.2	3 1/4	^a
2	4	No.3 stillborn No.4 exchange x 1	1:20	160	2,620	—	—	+	12.4	3.5	3	5.7
3	4	0	1:5	5	2,830	+	—	+	12.5	2.2	4	2.8
4	6	No.4 exchange x 1	1:10	80	3,990	+	++	+	14.8	3.4	3	4.7 ^b
5	3	0	1:10	20	4,830	+	+	+	18.1	2.6	6	5.6 ^c

^a 4 hours old 4.6; 9 hrs 5.8; 20 hrs 9.5. No exchange transfusion was made.

^b Exchange transfusion was repeated at 7 hours old.

^c 3 hours old 5.4. The rise in bilirubin was the indication for exchange transfusion.

TABLE 11. Bone marrow findings in Haemolytic disease of the newborn.

Case No.	'Stem cells'		Erythropoietic cells		Granulopoietic cells		Lymphocytes Diff. %
	Diff. %	L.I.	Diff. %	L.I.	Diff. %	L.I.	
1	1.9	.26	36.1	.59	44.3	.40	9.2
2	3.4	.15	29.9	.41	63.7	.26	3.1
3	9.9	.16	16.4	.41	53.6	.26	12.7
4	7.4	.31	36.3	.47	47.2	.19	6.2
5	2.4	.19	54.5	.40	35.3	.15	3.3
Range in healthy neonates	3-8	.10-.12	21-55	.49±.06	30-55	.28±.04	6-17

marrow specimens were obtained from the tibia before commencement of exchange transfusion. In Case 1 an exchange transfusion was planned but was postponed and later found not necessary.

Results

The main findings in the bone marrow differential counts of the five cases are given in Table 11, as are also the labelling indices of the 'stem cell' and differentiated proliferating pools. The values from a corresponding investiga-

tion of healthy neonates are also given. There appear to be no significant differences between affected and healthy infants with the exception of the labelling index of the 'stem cell' pool, which was significantly higher ($p < 0.005$) in the children with haemolytic disease.

By pooling the data from the differential counts of all five cases more reliable values of the relative proportions of the different cellular compartments within the erythropoietic and granulopoietic cell series were obtained (Table 12). The distribution

TABLE 12. Proportional distribution of erythropoietic and granulopoietic bone marrow cells in Haemolytic disease of the newborn. The size of each compartment is relative to compartment 1 (Er-1 and Gr-1).

Pooled data of	Erythropoietic cells					Granulopoietic cells			
	Er-P			Er-NP	NP/P	Gr-P		Gr-NP	NP/P
	Er-1	Er-2	Er-3	Er-4		Gr-1	Gr-2	Gr-3+4	
5 cases	1	8	14	25	1.2	1	1.6	7.8	3.0
60 healthy children	1	6	13	18	0.9	1	1.6	8.3	3.2

pattern was roughly the same as in healthy infants except for an over-representation of Er-4 in the affected infants. It is quite likely, however, that this can be accounted for by the inevitable contamination of marrow aspirates with peripheral blood which, in haemolytic disease of the newborn, contains an increased proportion of nucleated red cells, mainly Er-4. The ratios between the non-proliferating and the proliferating erythroblasts were, however, about the same.

The *mean grain counts* of the proliferating pools and compartments within erythropoiesis and granulopoiesis were within the normal range.

Discussion

All the five infants had haemolytic disease, caused by iso-immunization of the pregnant mother with consequent destruction of the red cells in the foetus and neonatally. In this disease, the haemolysis is more or less compensated by bone marrow hyperplasia and extensive extramedullary haemotopoiesis [103]. The results of this study confirmed the findings of Dillon and Krivit [38] and Hyman and Sturgeon [56] that in haemolytic disease the neonatal distribution of erythropoietic cells in bone marrow differential counts did not differ significantly from the normal. Nor were there any marked differences as regards the other main cell types.

The proportions of the successive compartments within the erythron appeared roughly normal, as were the

values of the labelling index of the proliferating erythropoietic pool. These data together indicate that the increased erythropoietic cell mass has an apparently normal proliferation and maturation schedule. No evidence was thus found for the proposal of Stohlmann et al. [108] that 'skipping' of terminal divisions occurs in acquired haemolytic anaemia. Any significant degree of intramedullary haemolysis is unlikely since it would require the improbable situation that each erythroblast type was affected in proportion to its relative frequency.

The relative size of the 'stem cell' pool varied somewhat more widely than normal but the material is too small for statistical evaluation. The labelling index was, however, considerably higher than that of healthy neonates, which means that a larger proportion of 'stem cells' was in the generative cell cycle. This indicates a more intense proliferation within the 'stem cell' pool. The increased erythropoietic cell mass thus results from a larger influx of cells from the hyperactive self-perpetuating 'stem cell' pool. Infants affected by haemolytic disease are exposed to a greater degree of tissue hypoxia than are normal infants, both before and after birth. Other investigators [4, 49, 60] have found elevated levels of erythropoietin (ESF) in plasma from infants with anaemic hypoxia due to haemolytic disease of the newborn. According to the current concept of the mechanism of its action (cf. p. 47), an elevated level of ESF induces an increased transformation of 'stem cells' into the erythroid cell line.

The increased cellular proliferation within the 'stem cell' pool found in these cases of haemolytic disease, is in good agreement with this concept. It provides evidence that the 'stem cell' pool, as defined in this study, seems to have functional 'stem cell' properties as expected of a self-maintaining cell pool.

To summarize, from the findings of

the present study it is proposed that haemolytic disease of the newborn is characterized by an apparently normal proliferation and maturation within an increased erythropoietic cell mass. The increased proliferation in the 'stem cell' pool is an ESF mediated response to an increased demand for new erythroid cells due to the high peripheral erythrocyte loss.

B. CONGENITAL HYPOPLASTIC ANAEMIA

Clinical material

Three infants with a diagnosis of Congenital (erythroid) hypoplastic anaemia were selected for study. They fulfilled the diagnostic criteria given by Diamond et al. [37] of: (1) normochromic, normocytic anaemia with onset in early infancy; (2) a specific deficiency of the erythroid elements of the bone marrow; and (3) normal leukopoiesis and thrombopoiesis.

The results of the present study refer to investigations of bone marrow specimens that were obtained within a few days after the patients' admission to the Paediatric Clinic, Uppsala, and prior to corticosteroid therapy.

The relevant clinical data are summarized below.

Case 1. J.P.B., male. He was the first child of healthy parents. There was no familial occurrence of haematological diseases. Pregnancy and delivery were uncomplicated but the infant was pale and flaccid after birth and he had also a small chin, large tongue, and cleft

palate. The birth weight was 2.6 kg and length 45 cm. Laboratory findings were on the first day: haemoglobin 5.4 g%; erythrocyte count 1.4 million/mm³; reticulocyte count 0.1 %; leucocyte, differential and platelet counts, normal. Blood types of both the mother and the infant were group 0, Rh(+). Coombs' direct test was negative. Blood transfusions were given to the infant on the second day of life and after 3 weeks and 6 weeks. The reticulocyte counts remained low while leucocytes and platelet counts were normal.

When the patient was 8 weeks old he was admitted to the Paediatric Clinic, Uppsala, for further study. It was again noted that he was pale but not icteric. Liver and spleen were just palpable. The main laboratory findings were similar to those neonatally. A bone marrow aspirate showed marked erythroid depression but was normal in other respects (Table 13). The results of the analysis of this marrow specimen are reported in detail below.

The child was given prednisolone orally and a reticulocytosis was manifest ten days later. His condition deteriorated, however, due to gastroenteritis and pneumonia. After recovery, reticulocytosis recurred and a small rise in haemoglobin values was noted, but later laboratory

data showed varying reticulocyte counts and falling haemoglobin values. Additional blood transfusions were given. The subsequent course was characterized by repeated attacks of unexplained fever. Electrophoretic pattern of serum proteins was normal (immunoglobulin pattern was not analysed). The patient deteriorated and died at the age of four and a half months. Autopsy revealed acute lobular pneumonia as the immediate cause of death. The bone marrow was hypoplastic with an erythroid aplasia and there was also moderate liver necrosis.

Case 2. M.J., male. He was the third child of healthy parents. There was no family history of blood dyscrasia. Pregnancy and delivery were uneventful. The birth weight was 4.0 kg. He was considered normal during the neonatal period, except for the presence of a double-thumb on his right hand. His weight gain was normal. Apart from vomiting for a few days at 4-5 weeks of age he was healthy. When he was 7 weeks old he was admitted to hospital because of a strangulated inguinal hernia which was reduced without operation. No peripheral blood study was made, but the mother stated later that the child was pale from that time on. At 8 weeks of age he appeared tired and feeble and had attacks of vomiting and moderately loose stools. After a few days he was admitted to hospital. His general condition was then poor and he was very pale but not icteric. The liver and spleen were not palpable. Peripheral blood values showed a pronounced anaemia with haemoglobin 1.5 g%. Reticulocyte count was 0.6 %, and leucocyte, differential and platelet counts were normal. Repeated blood transfusions were given during the following days and he improved considerably.

At 10 weeks of age he was admitted to the Paediatric Clinic, Uppsala, for further study. On physical examination he was within normal limits, except for pallor. Laboratory findings were: haemoglobin 8.7 g%; erythrocyte count 2.8 millions/

mm³; reticulocyte count 0.2 %. In other respects the blood picture was normal. Coombs' direct test was negative. A bone marrow specimen was analysed according to the methods used in the present study. The smear had a 'hypocellular' appearance and red cell precursors were almost absent while the other cell types appeared normal (Table 13).

Prednisolone administration was begun at 12 weeks of age. The haemoglobin was then 6.1 g%. There was a sharp rise in the reticulocyte count after two weeks and this therapeutic reaction was the same on repeated trials with prednisolone. The rise in haemoglobin value was less than expected, however, and reached the 8.0-9.0 g% level after four months of treatment. There were no signs of abnormal haemolysis. During this period hypocellular bone marrow specimens were obtained on repeated aspirations. At the time of writing, the child has required interrupted treatment with corticosteroids for two years. On two occasions the haemoglobin decreased to levels requiring blood transfusion.

Case 3. S.J., male. He was the first child of healthy parents. There was no family history of haematological diseases. Pregnancy and delivery were normal. The child weighed 3.6 kg at birth. He appeared healthy during the neonatal period and up to 8 weeks of age, when he was admitted to hospital because of pallor, which had probably been present for some time. On physical examination, apart from the pallor there was nothing abnormal. Laboratory findings were: haemoglobin 4.1 g%; reticulocyte count 3.1 %; leucocyte count 2,500/mm³ with differential values of 26 % neutrophils, 6 % eosinophils, 1 % basophils, 61 % lymphocytes, and 6 % monocytes, platelet count normal. The leucocyte and differential counts were normal on later occasions. He was given three blood transfusions during the following three weeks.

At three months of age he was ad-

TABLE 13. Bone marrow findings in Congenital hypoplastic anaemia.

Case No.	'Stem cells'		Erythropoietic cells		Granulopoietic cells		Lymphocytes
	Diff. %	L.I.	Diff. %	L.I.	Diff. %	L.I.	Diff. %
1	7.7	.35	5.3	.34	55.4	.31	24.3
2	7.3	.02(.10) ^a	0(0.3) ^a	.35	75.3	.21(.22) ^a	8.6
3	13.8	.36	4.8	.57	40.7	.33	28.8
Normal range	8-18	.27±.07	5-10	.49±.06	40-65	.28±.04	10-40

^a Figures in parenthesis refer to an analysis two weeks later.

mitted to the Paediatric Clinic, Uppsala, for further investigation. He was pale, but on examination appeared normal in other respects. Laboratory data showed: haemoglobin 9.4 g %; erythrocyte count 3.2 millions/mm³; reticulocyte count 1.0 %; leucocyte, differential and platelet counts, normal. A bone marrow aspirate was 'normocellular' but had a low proportion of erythroblasts while the other elements were normal (Table 13). Detailed results of differential counts and *in vitro*-labelling of this specimen are given below.

There was a gradual fall in haemoglobin to 7.4 g% during the following four weeks with reticulocyte counts around 1-2 %. There were no signs of abnormal haemolysis. The patient was thereafter given prednisolone intermittently and subsequent rises in reticulocyte counts and haemoglobin values were noted, followed by reticulocyte counts of 0.2-0.6 % when the therapy was discontinued. The child has now received intermittent corticosteroid therapy for one year and a half and no blood transfusions have been necessary.

Results

Peripheral blood data are given above in the presentation of the three

cases. The common pathological findings were marked anaemia and an absolute reticulocytopenia, the leucocyte, differential, and platelet counts being normal.

The bone marrow specimens of Cases 1 and 3 appeared to have normal cellularity. In Case 2 the marrow was 'hypocellular'; this was not an accidental occurrence as it was constantly found on repeated aspirations of bone marrow. In all cases the morphology of the individual bone marrow cells was normal.

The bone marrow findings are summarized in Table 13. A common finding in all marrow specimens was an erythroid hypoplasia. This was very pronounced in Case 2, where no erythroblast was found in the differential count of the first specimen, and in both the second and third aspirate there were only 3 erythroblasts per 1,000 nucleated cells. This meant that it was, for practical reasons, not possible in Cases 1 and 2 to calculate the erythropoietic labelling index from 200 proliferating erythroblasts, as in the

TABLE 14. *Proportional distribution of erythropoietic and granulopoietic bone marrow cells in Congenital hypoplastic anaemia. The size of each compartment is relative to compartment 1 (Er-1 and Gr-1).*

Case No.	Erythropoietic cells					Granulopoietic cells			
	Er-P				NP/P	Gr-P		Gr-NP	NP/P
	Er-1	Er-2	Er-3	Er-4		Gr-1	Gr-2	Gr-3+4	
1	1	2	1.4	1.7	0.53				
2	1	4.7	4.3	13.7	1.37				
3	1	2.9	4.1	4.8	0.57				
1 + 2 + 3 ^a	1	2.7	3.0	3.6	0.65	1	1.7	9.4	3.4
60 healthy children	1	6	13	18	0.9	1	1.6	8.3	3.2

^a Pooled data from differential counts of all three cases.

standardized method. Thus, in Case 1, the erythroid labelling index was calculated from a total of 96 proliferative cells and in Case 2 from 60 cells, found in several smears examined. Therefore, the values obtained in Cases 1 and 2 suffer from a larger uncertainty than in Case 3. The magnitude of certain changes found was, however, significant.

TABLE 15. *Labelling indices of proliferating erythroblasts in Congenital hypoplastic anaemia.*

Case No.	Erythropoietic cells			
	Er-1	Er-2	Er-3	Er-P
1	.50	.35	.23	.34
2	.67	.43	.21	.35
3	.64	.66	.50	.57
1 + 2 + 3 ^a	.58	.52	.39	.48
60 healthy children	.52	.63	.42	.49

^a Pooled labelling data of all three cases.

Differential counts showed that the proportional distribution of erythroblasts within the erythron was abnormal (Table 14). Compared with healthy infants there was a reduction in all the erythropoietic compartments, the more mature the cell the greater being the decrease. The proportional distribution in Case 2 was not as abnormal as that of Cases 1 and 3 although it was still anomalous. The calculated values for Case 2 have, however, less significance due to the small number of cells found. The corresponding values for the granulopoietic cells were within normal limits.

The *labelling indices* of both the proliferating erythroid pool and compartments are given in Table 15. While the index of the pool of Case 3 was normal, in Cases 1 and 2 it was apparently subnormal as were the values for compartment Er-3. The labelling indices of individual compartments are

liable to some uncertainty due to the problem of correct classification of the different cell types. In Cases 1 and 2 the small number of proliferating erythroblasts found increases this uncertainty and the labelling indices calculated for these cases are therefore less reliable than those of Case 3.

Tables 14 and 15 also give the values of the proportional distributions and labelling indices of erythroblasts calculated by combining together the values from the three cases. There was no significant difference between the proportions of Er-2, Er-3 and Er-4, in contradistinction to the normal increase in consecutive compartments. The labelling indices calculated in the same way were not significantly different from those of healthy children.

The *mean grain count* of the proliferating erythroid pool in congenital hypoplastic anaemia was higher (45 grains) than normal (30 grains). This difference was evidently due to the larger proportion of immature erythroblasts in the pool of the anaemic cases.

Apart from the erythropoietic series, the other cell lines appeared normal in Cases 1 and 3. In Case 2, 75 % of the population of the marrow aspirate consisted of neutrophilic granulopoietic cells due to a large proportion of mature granulocytes. The granulopoietic labelling index was within normal limits in all three cases. The labelling index of the 'stem cell' pool was normal in Cases 1 and 3 but significantly lower in Case 2 (.02 and .10 in two aspirates, compared with the normal values at that age $.27 \pm .07$).

Discussion

The pathogenesis of Congenital hypoplastic anaemia is unknown and various mechanisms have been proposed [37]. The etiology is possibly not the same in all cases of the disease. Cases 1 and 2 had other congenital anomalies, suggesting more generalized constitutional damage. There was no evidence of depression of erythropoiesis due to blood group antibodies or toxic substances or of thymoma in any of the cases. Some evidence has been obtained that a metabolic defect in tryptophane metabolism may be present in some cases involving some pathway essential to the production of an erythrocyte precursor [37]. A tryptophane loading test was performed on Case 2 but was normal. It has also been proposed [108] that at birth there is a changeover to a different regulatory mechanism of erythropoiesis than during foetal life; this mechanism is supposed to be disturbed in this disease.

In all three cases the essential criterion of erythroid hypoplasia of the bone marrow was present. This indicates that the mechanism of the disease apparently involves essential processes in the differentiation of 'stem cells' to erythroblasts. As regards the proportional distribution of erythroid compartments there is some divergence of opinion. It has been proposed that the defect is due to a hindered influx from the 'stem cell' pool, the erythron itself being normal [96]. Some investigators, on the other hand, consider that most of the eryth-

roid cells are young proerythroblasts, subsequent development being blocked [37, 102]. A maturation disturbance in orthochromatic erythroblasts has also been claimed [85]. Dechamps et al. [34] review the question of 'maturation stops' which have been claimed as occurring at various stages of red cell development.

A consistent finding in the present study was an abnormal distribution pattern with an obvious reduction of more mature compartments indicating a decreased flow through the erythron. This finding can be interpreted as evidence of 'skipped divisions', i.e. some reticulocytes are produced directly from earlier stages rather than from the orthochromatic erythroblasts, or of 'ineffective' erythropoiesis, i.e. some cells die before reaching maturity. The first alternative with a maturation of erythroblasts from Er-1 to Er-4 without intervening cell divisions is not probable. If the erythroblasts did not synthesize DNA during this maturation phase, the labelling index would have been markedly reduced, and if there had been DNA-synthesis without succeeding divisions, polyploid erythroblasts with abnormal morphology would probably have been found. Further, it would cause a production of large erythrocytes, i.e. macrocytes, but in all three cases the erythrocytes were mainly of normal size.

Thus, cell death, i.e. loss, within the erythron remains as the most probable explanation of the reduction of the erythropoietic cell flow noted. Due to the relatively few number of erythro-

blasts found in Cases 1 and 2, it was not possible to ascertain at which generation phase this cell loss is located. However, it seems likely that such a loss should occur mainly in G_1 phase, because of the following reasons. The erythropoietic labelling index was apparently subnormal in two cases, and normal in one case. In the labelling experiments the incubation period was so short that it seems reasonable to assume that any cells which took up thymidine would not have time to die, disintegrate and disappear and thus give a false low value for the labelling index. Death of nucleated cells from the erythron at the end of the G_1 phase would cause a moderate decrease of the labelling index while loss at the end of the S phase should result in an elevation. Loss in late G_2 phase would give a normal labelling index and a normal distribution of Er-2 which is not consistent with the data.

The findings of the present study justify the concept that the main pathogenetic mechanism is a decrease in the differentiation of 'stem cells' into the erythron but indicate also a disturbance within the erythron itself. ESF exerts its main effect on the differentiation of 'stem cells' but probably has also some influence on the erythron (cf. p. 47). The pathophysiological disturbances appear to be those expected from either a deficiency of ESF or a blocking of its effect. The latter is more likely as an elevated serum ESF level is a frequent finding in congenital hypoplastic anaemia [50]. It has been proposed that an autoim-

mune mechanism may be responsible for the development of erythroid, hypoplastic anaemia in adults [5] partly because of the frequently noted association with thymoma. No cases of thymoma in congenital hypoplastic anaemia have been reported but this does not exclude an autoimmune mechanism. Schooley and Garcia [99] reduced erythropoiesis in mice by means of an antibody against ESF, which supports the hypothesis of a possible autoimmune mechanism. The therapeutic effect of corticosteroids would then be explained as the reversal of an antibody-antigen reaction, thus liberating active ESF. Although termed 'congenital' because of the early age of onset, this disease is seldom diagnosed in infants below one month old [37]. A possible autoimmune mechanism is then either not present in foetal life or is suppressed by the protective action of transplacentally transported maternal ESF that is not affected by the autoimmune reaction.

If the above concept is correct, it implies that ESF is essential not only for the transformation of 'stem cells' but also for the development of differentiated stages (cf. [17]).

Case 2 differed from the other two cases in that (1) hypocellular bone marrow specimens were obtained in repeated aspirations, (2) the labelling index of the 'stem cell' pool was subnormal, and (3) though corticosteroids induced reticulocytosis the rise in haemoglobin concentration was smaller than expected. The two first differences indicate a low cellular proliferation in a 'stem cell' pool of probably reduced absolute size. The effect of increased differentiation of 'stem cells' into erythroid cells would, therefore, be relatively modest, at least in the beginning of the course of therapy. Additional pathogenetic mechanisms may also interfere, such as a production of short-lived reticulocytes that are defective in some respect(s) (cf. 'stress reticulocytes' [118]).

C. INFANTILE GENETIC AGRANULOCYTOSIS

C. Infantile genetic agranulocytosis

In 1956 Kostmann [65] reported what he considered to be a new disease in infancy. During a period of 4 years he observed, in an isolated district in Northern Sweden, six children who presented a clinical picture with certain common features. Histories of eight additional children who had the clinical criteria of the condition were found among local records over a 22 year period. The main features of the condi-

tion were: a history of skin infections from early infancy usually leading to eventual death from sepsis which were presumed to be due to the persistent and severe neutropenia with 'arrest' of neutrophil granulopoiesis in bone marrow which was found in all cases. Genetic evidence indicated that the condition was inherited as an autosomal recessive, the high incidence in the population concerned being due to a high incidence of intermarriage.

Kostmann suggested the disease be called *Infantile genetic agranulocytosis* (I.G.A.) and subsequently a number of cases with similar features have been reported in other localities in various parts of the world (reviewed in [76]). The pathogenesis is not known. Hitherto no studies of the kinetics of the neutrophilic cell series in I.G.A. have been published, and this was the aim of the present investigation.

Clinical material

In the last few years three patients who fulfilled the criteria of I.G.A. were warded at the Paediatric Clinic, Uppsala. They were all females, two of them being sisters (Cases 1 and 2). The parents of the two sisters lived in the same district as Kostmann found most of his cases, and their forebears had dwelt there for several generations. The parents of the third case lived in another part of Northern Sweden, but it appeared that forebears of both of them had also lived in the I.G.A. area. There was no known consanguinity between the parents in either of the families but the mother of Cases 1 and 2 and the father of Case 3 may be distantly related to each other. There is a probable history of I.G.A. in both families as their pedigrees indicate that some infants died from infectious disease during early infancy.

Cases 1 and 2 were the first and second children, respectively, of healthy parents. Three years before her marriage the mother had given birth to a daughter (by another father) who

was quite healthy at the time of the investigation of Case 2.

Case 3 was the second child of healthy parents. She had a brother three years older who had been healthy up to the time of the investigation of his sister.

Clinical data of the three children are summarized below.

Case 1, M.V. Pregnancy and delivery were normal. The child weighed 3.0 kg at birth. She appeared healthy up to 4 weeks of age when she was admitted to a local hospital because of fever and a purulent skin infection around the site of a B.C.G.-vaccination on her left thigh. She had also some minor skin infections elsewhere. On physical examination nothing abnormal was found except for the skin infections. These healed within three weeks by incision and antibiotic therapy. The peripheral blood picture was normal, except for constant abnormal leucocyte differential counts with pronounced neutropenia and marked monocytosis. A bone marrow smear showed an apparent lack of neutrophils of all maturation stages but was difficult to interpret due to heavy peripheral blood admixture. At three months of age she was again admitted to hospital because of purulent skin infections. Her general condition was, however, good and the laboratory findings were, in the main, the same as during the first period.

Two weeks later the infection still persisted and she was then transferred to the Paediatric Clinic, Uppsala for further investigation. Apart from a few purulent skin areas, physical examination revealed no abnormality. The haematological data are given in Table 16 and show a virtual agranulocytosis. The results of the analysis of a bone marrow aspirate are given in Tables 17-19 and will be discussed later in detail.

She was then sent back to the local

hospital. A therapeutic trial with corticosteroids and testosterone had no effect on the white blood cell picture. Her condition deteriorated with recurrent fever and apparent septicaemia in spite of intense therapy and she died at the age of five months. The parents refused autopsy.

Case 2. H.V. (Younger sister of Case 1.) Pregnancy and delivery were uncomplicated. Her birth weight was 2.9 kg. Because of her sister's death in I.G.A., she was admitted to a local children's hospital soon after birth. On physical examination there was nothing abnormal. A blood count at twentyfour hours old showed: haemoglobin 18.4 g%; reticulocytes 7.2 %; leucocytes 9,200/mm³ with a differential count of neutrophils 18 % (1,656/mm³), eosinophils 1 %, lymphocytes 72 %, and monocytes 9 %. Two days later there were 6,100 leucocytes/mm³ with 13 % neutrophils (793/mm³), 1 % eosinophils, 44 % lymphocytes, and 42 % monocytes.

When 12 days old, she was transferred to the Paediatric Clinic, Uppsala, for further investigation. Physical examination was again noted to be normal, as were also the laboratory findings except for neutropenia (5 %, 440/mm³) and monocytosis (40 %, 3,520/mm³) in peripheral blood. She was kept in hospital for 7 months and her general condition remained good, and physical and mental growth was normal. An almost complete agranulocytosis was the main feature of the disease with 0-2 % neutrophils in the differential count and a persistent monocytosis (Table 16). Repeated bone marrow aspirates were analyzed and the results are discussed in detail below. Therapeutic trials with plasma infusions, somatotrophic hormone, and repeated series of pyrogen injections (Pyrexal®) had no measurable effect upon the white cell picture. While in hospital she had skin infections on three occasions, and a purulent otitis media once, but was in good condition when she was sent home.

On continuous antibiotic therapy she remained in good health, in spite of persistent agranulocytosis, until the age of twelve months, when she had a perianal abscess. This healed, but two months later she became seriously ill with a febrile gastroenteritis that developed into a fatal peritonitis in spite of intense treatment. The parents again refused autopsy.

Case 3. M.E. Normal full-term delivery; birth-weight 3.2 kg. Nothing abnormal was noted until five weeks of age when she was admitted to a local hospital because of otitis media and pyelitis. A blood count showed normal haemoglobin concentration, leucocytes 7,900/mm³ and a differential count with neutrophils 2 % (158/mm³), lymphocytes 88 %, and monocytes 10 %. Her condition deteriorated and a perirenal abscess developed, but this appeared to heal after incision and intense antibiotic treatment. A moderately severe anaemia developed, the thrombocyte count was normal, and the marked neutropenia persisted.

At three months old she was transferred to the Paediatric Clinic, Uppsala, for further investigation. She was then in a good general condition, physical examination revealing no abnormality except for a slight inflammatory reaction around the site of the perirenal incision. On admission there was complete absence of neutrophils and this agranulocytosis remained during the five months of her stay in hospital (0-2 % neutrophils). The bone marrow aspirate taken at four months of age is described in detail below. Pyrogen injections (Pyrexal®) and somatotrophic hormone were tried therapeutically but without noticeable effect. The perirenal abscess recurred and she had a new outbreak of skin infections but was otherwise in good condition during her stay in hospital.

After leaving hospital she has remained at home under continuous antibiotic therapy. At 14 and 17 months of age she

TABLE 16. *Haematological data in three cases of Infantile genetic agranulocytosis.*

Case No.	Age (month)	Haemoglobin (g %)	Reticulocytes (%)	Platelets (per mm ³)	Total leucocytes (per mm ³)	Differential count (%)					Sedimentation rate (mm/hr)
						Neutrophils	Eosinophils	Basophils	Mono-cytes	Lymphocytes	
1. M.V.	4	10.2	5.2	556,000	10,900	0.5	1	1	13	84.5	21
2. H.V.	$\frac{1}{2}$	18.6	1.8	330,000	8,400	0.5	1.5	—	9.5	88.5	0
	1	15.0	1.8	319,000	5,900	3	8	—	15	74	—
	4	9.9	2.4	250,000	11,000	1	1.5	—	33.5	64	50
3. M.E.	6	12.6	1.6	315,000	8,100	0.5	0.5	0.5	12	86.5	12

was temporarily admitted to a local hospital because of febrile throat affections. At the time of writing she is 17 months old and remains in good health although her peripheral blood is still agranulocytic.

The pattern of the serum immunoglobulins was normal in all three infants.

Results

The peripheral blood data are given

in Table 16. These were obtained at the same time as the bone marrow analysis.

The bone marrow findings are summarized in Table 17. The smears showed normal cellularity and the morphology of the different cell types was normal.

The bone marrow differential counts

TABLE 17. *Bone marrow findings in three cases of Infantile genetic agranulocytosis.*

Case No.	Age (month)	'Stem cells'		Erythropoietic cells		Granulopoietic cells		Mono-cytes	Lymphocytes
		Diff.%	L.I.	Diff.%	L.I.	Diff.%	L.I.	Diff.%	Diff.%
1. M.V.	4	11.0	.29	12.3	.50	4.9	.18	2.5	34.4
2. H.V.	$\frac{1}{2}$	33.7	.03	1.8	.53	14.2	.27	1.2	12.9
	1	13.7	.34	10.8	.68	11.0	.21	1.1	32.2
	4	8.8	.36	6.7	.54	21.7	.28	2.5	49.0
3. M.E.	6	10.8	.35	16.9	.57	4.5	.28	4.4	57.7
Normal range	$\frac{1}{2}$		.10—.30						
	1—6		.20—.40						5—30
	0—12	8—18		5—15	.49±.06	40—65	.28±.04	1—5	1—4

TABLE 18. *Proportional distribution of granulopoietic and erythropoietic bone marrow cells in three cases of Infantile genetic agranulocytosis. The size of each compartment is relative to compartment 1 (Gr-1 and Er-1). Percentage distribution of Gr-1 is given within brackets.*

Case No.	Age (month)	Granulopoietic cells				Erythropoietic cells				NP/P
		Gr-P		Gr-NP	NP/P	Er-P			Er-NP	
		Gr-1	Gr-2	Gr-3+4		Er-1	Er-2	Er-3	Er-4	
1. M.V.	4	1 (2.6)	0.31	0.58	0.4					
2. H.V.	$\frac{1}{2}$	1 (2.6)	0.85	3.65	2.0					
	1	1 (3.3)	0.79	1.55	0.9					
	4	1 (6.1)	1.16	1.39	0.6					
3. M.E.	6	1 (3.7)	0.19	0.03	0.02					
1 + 2 + 3 ^a		1 (3.7) ^b	0.7	1.4	0.8	1	6	13	18	0.9
53 healthy children		1 (5.2) ^b	1.6	8.3	3.2	1	5	13	16	0.8

^a Pooled data from differential counts of all three cases.
^b Mean value.

showed certain characteristic features, the most notable being the reduction of mature neutrophils. As regards the other cell lines, the proportional distributions of erythropoietic cells and eosinophils were normal. The 'stem cell' pool was of normal relative size except for the high value found in Case 2 at the age of $\frac{1}{2}$ month. At this age also the frequency of monocytes was rather high (24%). The proportion of monocytes was, in fact, higher than normal in Cases 1 and 2. The lymphocyte percentage was high in

Case 2 at the age of 4 months and in Case 3, and normal in Case 1 and in Case 2 at $\frac{1}{2}$ and at 1 month of age. Neutrophilic granulocytes were very rare in all marrow specimens. Table 18 gives the proportional distribution of the granulopoietic cells and the main deficit appears to be of mature non-proliferating neutrophils (Gr-3 + 4). Within the proliferating pool the promyelocyte (Gr-1) values are only moderately below normal, while the myelocyte (Gr-2) values are markedly depressed. The smallest divergence

from normal was found in Case 2 (1/2 month) and the largest in Case 3. The ratio Gr-NP/P decreased with increasing age. In comparison, the proportional distribution of the erythropoietic cells was quite normal.

The *labelling index* of the proliferating erythroid pool was within the normal range except for an elevated value in Case 2 (1 month). The 'stem cell' pool had a low labelling index in Case 2 (1/2 month) but normal values in the remaining instances. Immature monocytes were constantly found and exhibited varying degrees of labelling. They were too few in number, however, to allow any conclusions about their labelling pattern.

The labelling data of the granulopoietic cells are given in detail in Table 19. The labelling index of the proliferating pool was very low in Case 1 and moderately low in Case 2 (1

month) but otherwise normal. The promyelocyte and myelocyte compartments were analysed by counting about 400 proliferative cells. Compared with normal control material low values were found for the same two instances as in the case of the low labelling index of the pool. The labelling index of the myelocyte compartment was low also in Case 3.

The *mean grain counts* of the proliferating pool and proliferating cell types within the granulopoietic series were not abnormal.

Discussion

The three cases described in this section fulfil, both in their clinical course and laboratory data, Kostmann's criteria of Infantile genetic agranulocytosis.

The blood and bone marrow pictures were similar in all three cases. The typical blood film revealed an extreme paucity of neutrophils and an excess of monocytes, the other cell types occurring in fairly normal numbers. A moderate eosinophilia was observed occasionally. A moderate anaemia developed in Cases 1 and 2 during the investigation and it seems likely that this can be attributed to the repeated infections. No platelet abnormality was found.

The cellularity of the bone marrow samples investigated was approximately normal. Changes in the differential counts probably reflect changes in the absolute numbers of different cells because of the following reasons: Firstly, the 'normal' distribution of erythroid

TABLE 19. *Labelling indices of granulopoietic bone marrow cells in Infantile genetic agranulocytosis.*

Case No.	Age (month)	Granulopoietic cells		
		Gr-1	Gr-2	Gr-P
1. M.V.	4	.20	.12	.18
2. H.V.	1/2	.31	.23	.27
	1	.25	.18	.21
	4	.33	.21	.28
3. M.E.	6	.29	.17	.28
1 + 2 + 3 ^a		.28	.19	.25
53 healthy children		.32	.25	.28

^a Pooled labelling data of all three cases.

precursors probably corresponded to a normal total mass of the erythroid precursors since the patients were able to maintain rather normal red blood counts and haemoglobin levels without undue reticulocytosis and, secondly, the increased proportion of immature monocytes in the bone marrow smears is reflected in the monocytosis in peripheral blood.

The most characteristic findings in the bone marrow smears were the great reduction in the neutrophilic cell series and the increase of monocytes. The largest decrease in the neutrophil series was that of the mature granulocytes. Myelocytes had also lower than normal values while the promyelocytes were not significantly reduced.

These findings corroborate and extend previous studies with respect to bone marrow cellularity and differential count [65, 76]. In contrast, however, to the findings of MacGillivray et al. [76] there was no eosinophilia in the marrow. There was a tendency for the labelling indices of the granulopoietic cell series to be low, this being more marked for myelocytes than for promyelocytes and most apparent in Case 1.

It seems reasonable therefore to draw the following conclusions.

(1) The 'stem cell' pool is normal in size and proliferative activity.

(2) The cell flow from the 'stem cell' pool to the granulopoietic cell line is normal because the frequency and labelling index of promyelocytes was not significantly abnormal.

(3) The production of mature types

from immature differentiated cells is markedly reduced.

As discussed in Chapter III changes in the relative proportions of compartments must be interpreted with caution, a smaller compartment can be a result of either a reduced influx from the preceding compartment, or a shorter transit time through the compartment. The length of the S phase in mammalian cells in both normal and pathological states appears to be rather constant [11]. Assuming that it is unchanged in I.G.A. the nearly normal labelling indices indicate nearly normal transit times. In I.G.A., in contrast to normal marrow, Gr-2 is smaller than Gr-1 and the non-proliferating pool Gr3+4 is very much smaller (Table 18). This pattern in I.G.A. is most likely due to death of cells in the proliferating pool, i.e. 'ineffective' granulopoiesis, while the surviving cells proliferate apparently normally. The alternative of greatly reduced proliferation must however be considered. Maturation in the granulopoietic series might occur by amitotic transformations. If cells undergoing such transformations maintained, as seems unlikely, a normal morphology, the labelling indices would be depressed since they would not take up ^3H -thymidine. Cells maturing amitotically might, on the other hand, have some DNA-synthesis without subsequent division and this would probably result in polyploid cells with an abnormal morphology.

Fig. 17 is a schematic model of the granulopoietic cell line indicating the effects of cell death in different gene-

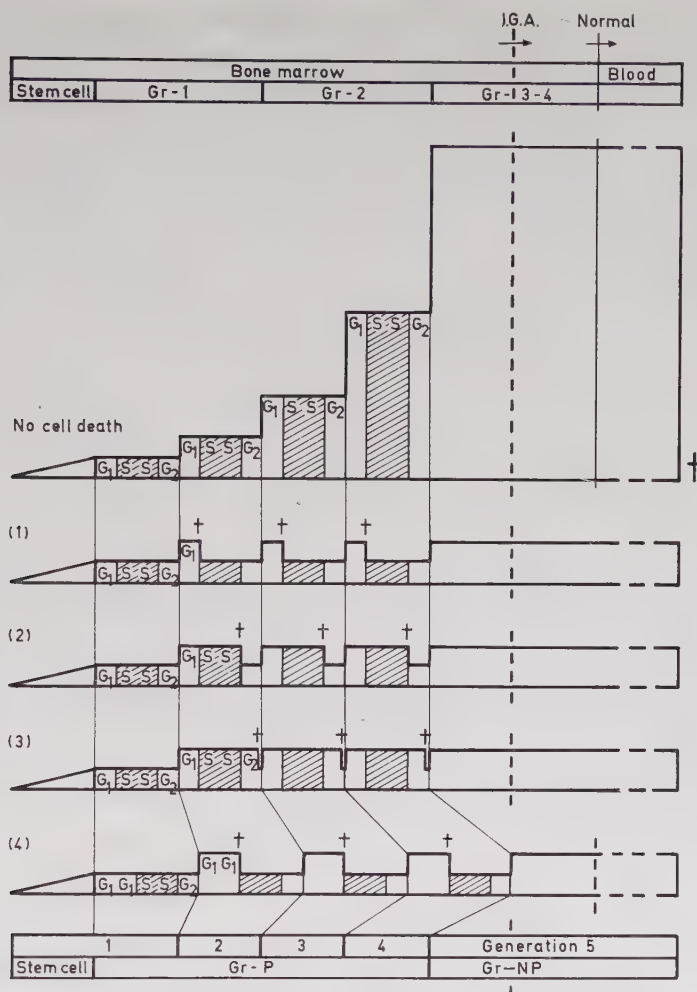


Fig. 17. The granulopoietic pool in Infantile genetic agranulocytosis (I.G.A.). A schematic outline of the effect on the sizes and labelling indices of pool and compartments due to cell death ($\dagger$) in different generative phases. The diagrams are drawn on the assumption that there are two generations of rather similar durations in each of the proliferating compartments, i.e. promyelocytes (Gr-1) and myelocytes (Gr-2). An earlier than normal release of granulocytes is indicated by the dashed line (I.G.A.). The diagram 'No cell death' indicates the normal healthy situation. The four alternatives show the effects of a 50% cell death at the end of (1) the G_1 phases, (2) the S phases, (3) the G_2 phases, and (4) prolonged G_1 phases. Table 20 gives the estimated values of the proportional distributions and the ratios between non-proliferating and proliferating cells (Gr-NP/P) and the possible changes in the pool labelling index that were calculated from the model. The best agreement between the I.G.A. data and the model appears to be with alternative (1), as is further discussed in the text. — Co-ordinates as in Fig. 13.

TABLE 20. *Effects of cell death in different phases of the generative cell cycle in the schematic model of granulopoiesis, described in Fig 14.*

State	Proportional distribution			Gr—NP/P	L.I.
	Gr—1	Gr—2	Gr—3 + 4		
Healthy children	3	5.1	25	3.2	normal
I.G.A.	2.4	1.7	3.4	0.8	(↘) ^a
Model					
No cell death	3	12	32	2.2	normal
Cell death at the end of					
(1) G ₁	2.25	2.5	2	0.4	(↘)
(2) S	2.75	3.5	2	0.3	(↗)
(3) G ₂	3	4	2	0.3	normal
(4) Prolonged G ₁	3	3.5	2	0.3	(↘)

^a The direction of the arrow under L.I. (labelling index) indicates the direction of change.

rative phases. The model is, of course, a gross simplification of the kinetics of granulopoiesis, many details of which are still unknown but the basic values (row “No cell death” in Table 20) are calculated on the assumption that there are two generations with rather similar transit times in each of the proliferating compartments Gr-1 and Gr-2.

Thus in Gr-1, there is one cell unit in generation 1 and two cell units in generation 2 since mitosis has occurred, i.e. 3 cell units in all. Similarly, Gr-2 will receive 4 cells from Gr-1 since entry into Gr-2 occurs after mitosis of generation 2 in Gr-1. With a subsequent mitosis in Gr-2, giving 8 cells in generation 3, the total number of cells in Gr-2 will be 12. Influx into Gr-3 from Gr-2 is determined by mitosis and thus 16 cells are derived from the 8 cells of generation 4 in Gr-2. Once in Gr-3 further maturation is amitotic so that the numbers of cells in Gr-3 and 4 are equal, i.e. 16 in each, a total of 32. In generation 1 are indicated the successive phases of a generative cell cycle up to mitosis, i.e. G₁—S—G₂, each phase schematically occupying one

fourth, two fourths, and one fourth, respectively, of a cell generation. The effects of cell loss, i.e. death, at various points are also shown in Fig. 17, and the calculated values from the models are given in Table 20. For example, row “(1)G₁” shows the effects of a 50 % cell loss in phase G₁ of the second, third, and fourth generations (death in the first generation would of course eliminate the entire production).

There is apparently no reserve of mature granulocytes in the marrow as judged from the differential count and the non-response to the pyrogen test [28]. Therefore either granulocytes die in the marrow or are released into the circulation earlier than normally in I.G.A. This is indicated schematically in Fig. 17.

The best agreement between the I.G.A. data and the model appears to be with cell death at the end of the G₁ phase, since loss at the end of the S phase would probably increase the labelling index while loss at the end of G₂ phase would probably leave it unchanged. Cell death in a prolonged G₁

phase (Fig. 17 (4) and row "(4) Prolonged G_1 " in Table 20) is also possible although even a moderate prolongation causes decrease of the labelling index. The proportional distribution will tend to be more normal, the later the time of death in the generation cycle.

It therefore seems plausible that 'stem cell' differentiation into the neutrophilic cell line is normal, the defect occurring at a later stage. It seems not unlikely that the viability may be reduced in the G_1 phases of the differentiated generations. This might be due to inability to synthesize DNA. Cells which survive presumably pass normally through S and G_2 phases and then divide. The proportion of cells destroyed in each generation should be at least 50 %, in order to explain the experimental finding that Gr-1 and Gr-2 are fairly equal in size. Since nor-

mally there is a large increase in size of successive compartments the final result is a markedly reduced production of mature neutrophils.

The etiology of this supposed disturbance of cellular metabolism remains obscure. Kostmann suggested on the basis of the study of a single case by Plum [65] that the cause was a congenital inability to utilize S-containing amino-acids. Treatment with cysteine had no effect, however. All the known haematinics have been tried for this condition without any observable benefit [76]. A deficiency of a plasma factor has been suggested, as transfusion of fresh plasma caused a temporary rise of neutrophils in one of two cases resembling I.G.A. reported by Bjure et al. [12]. Case 2 of the present study was given transfusions of fresh plasma but without effect.

D. PANCYTOPENIA

Pancytopenia, i.e. a severe deficit of erythrocytes, granulocytes and thrombocytes in circulating blood, may be of diverse etiology [115]. When it is associated with a hypocellular marrow, the term 'aplastic anaemia' is used. However, pancytopenia may also be associated with various degrees of increased cellularity, with a high proportion of differentiated cells in the marrow. The term 'primary, refractory anaemia' is preferred by Wintrobe [115] to indicate pancytopenia with various degrees of bone marrow cellularity.

The three patients of the present study all had one common feature—a severe pancytopenia. From other standpoints, however, they were heterogeneous, especially with regard to bone marrow histology and cytology. Their relevant clinical data are summarized below.

Clinical material

Case 1, J.M. was a boy aged 11 years. There was no family history of blood dyscrasia. He had appeared healthy apart from a tendency to nose-bleeding and bruising which became markedly exacerbated in May 1963. There was no known

exposure to agents toxic to bone marrow. He was admitted to a local hospital because of profuse nose-bleeding. The initial blood findings were: Hb 7.4 g%, leucocytes 4,300/mm³ with normal differential count, and platelets 35,000/mm³. Bone marrow puncture showed active erythropoiesis with signs of disturbed maturation, normal granulopoiesis and rather few megakaryocytes. There were no signs of abnormal haemolysis. Thrombocytopenic purpura was tentatively diagnosed and he was given repeated blood transfusions and then referred to the local children's hospital where he was put on corticosteroids for four weeks which were, however, without effect on the platelet value. Laboratory data during this period showed: haemoglobin about 10 g%, reticulocytes 0.2–0.8 %, leucocytes 5,000–7,000 with differential values of 20–30 % neutrophils, and platelets 50,000–60,000/mm³.

In July 1963 there was a serious fall in the haemoglobin value although there was no manifest bleeding and he was admitted to the Paediatric Clinic, Uppsala, for further investigation. Immediate blood transfusion had to be given because of the severe anaemia (Hb 4.9 g%). There were no lymphatic glandular enlargement, hepatosplenomegaly or malformations noted. Further examinations revealed a pronounced thrombocytopenia (< 10,000/mm³) and a manifest neutropenia (700/mm³). A bone marrow puncture yielded a fatty hypocellular aspirate, most of the cells being lymphocytes. 'Aplastic anaemia' was thus diagnosed and he was put on combined testosterone and prednisolone therapy [101]. The subsequent course was characterized by severe pancytopenia and recurring septic infections. There was a complete lack of therapeutic response and he had to be given repeated blood transfusions. A bone marrow aspirate after two months of therapy was extremely hypoplastic. In October 1963 he had massive intestinal bleeding and died. Autopsy revealed

multiple haemorrhages, localised infections and a hypocellular, fatty bone marrow. There were, however, small scattered areas in the marrow with signs of more increased haematopoietic activity, although these did not reach normal cellularity.

Case 2, E.T., a girl aged 3 years, was healthy until a tendency to bruising was noted in December 1962 at 2½ years of age. On investigation in February 1963 a low platelet count (16,000/mm³) and a moderate anaemia (Hb 9.3 g%) were found. No leucocyte or differential counts were made. Her condition was diagnosed as idiopathic thrombocytopenia and she was given iron. During the following months she became pale and tired but had no obvious bleeding.

In April 1963 she was transferred to the Paediatric Clinic, Uppsala, from the Orthopaedic Clinic, where she had been sent because of flat-feet. On examination she was very pale. Lymph glands, spleen, and liver were not enlarged. Blood examination showed pancytopenia: Hb 3.4 g%; erythrocytes 0.9 millions/mm³; reticulocytes 1.4 %; leucocytes 5,200/mm³ with a differential count of 8.5 % neutrophils, 90 % lymphocytes, and 1.5 % monocytes; platelets 10,000/mm³. A bone marrow aspirate was somewhat hypocellular with an increased proportion of lymphocytes and signs of disturbed maturation in the differentiated cell lines. (The detailed results are given below.) There was no familial occurrence of haematological disease. No congenital anomalies were found. However, on two previous occasions, September 1961 and September 1962, she had been given chloramphenicol for upper respiratory infections (3.75 g and 6.25 g, respectively) which seemed to be the likely cause of the 'aplastic anaemia'.

Combined treatment with testosterone and prednisone was started and after one month of treatment she was sent back to the local hospital. No therapeutic effect had been noted but only one blood

transfusion had been necessary. However, after three months of therapy the haemoglobin value began to rise. After four months, leucocyte and differential counts became normal, and after 11 months there was an obvious rise in the platelet count. The combined treatment was discontinued after 12 months, and subsequently the blood picture has remained normal (latest examination: March, 1966).

Case 3, Y.H., a girl 7 years old. There was no familial history of haematological disease, and no known exposure to toxic drugs or agents, except for chloramphenicol, as mentioned below. From her early infancy she had a tendency to recurrent upper respiratory infections and had been treated with various antibiotics, with, at least on one occasion, chloramphenicol. She also suffered from recurrent nose-bleeding and in March 1962, aged 5½ years, she was admitted to the local children's hospital because of profuse epistaxis. Her haemoglobin value was then 4.8 g%. Further blood findings were: leucocytes 10,400/mm³ with a differential count of 4 % metamyelocytes, 14 % band cells, 31 % segmented neutrophils, 29 % lymphocytes, and 22 % monocytes; 17 nucleated red cells/100 leucocytes; and platelets normal. A blood transfusion was given. No pathological reason for the recurrent nose-bleeding was found. Later a tendency to bruising appeared but on examination in July 1962 the platelet count was normal and a bone marrow smear showed only signs of iron deficiency. However, in February 1963, in addition to anaemia, she had also an obvious thrombocytopenia and neutropenia (300–600/mm³). At the same time nucleated red cells and myelocytes and metamyelocytes were found in the peripheral blood. A bone marrow aspirate was normocellular with abundant megakaryocytes, many of which were abnormal. There were signs of maturation disturbance in granulocytopoiesis with few mature

granulocytes. No signs of abnormal haemolysis were found. Her severe pancytopenia persisted and she suffered from recurrent infections. Treatment with ACTH had no effect.

In May 1963 she was admitted to the Paediatric Clinic, Uppsala, for further investigation. Examination revealed suppurative otitis, advanced dental caries, and a slight enlargement of the liver but the findings were otherwise normal and no congenital malformations were noted. The haematological data were: Hb 8.4 g%; erythrocytes 2.7 millions/mm³; reticulocytes 0 %; leucocytes 1,300/mm³, consisting mainly of lymphocytes; and platelets < 10,000/mm³. The bone marrow picture was essentially the same as on the previous occasion except for a megaloblastoid change in erythropoiesis and the presence of abnormally large thrombocytes. Infected dental foci were cleared but she still had recurrent septic infections. Her pancytopenia persisted with reticulocyte counts of about 2 %. Neither normal nor clearly leukaemic blast cells were ever found in the peripheral blood. Therapeutic trials with vitamin B₁₂ and folic acid were unsuccessful, as also was treatment for three months with testosterone and prednisolone. A bone marrow aspirate in September 1963, after two months of hormone treatment, was somewhat 'hypercellular' but otherwise appeared the same as on the previous occasions. She had to stay in hospital permanently because of repeated gastro-intestinal bleeding, which required frequent blood transfusions. At the end of October 1963 she died in the local hospital after an acute intestinal haemorrhage. Autopsy revealed a very cellular bone marrow packed with immature blast cells which was interpreted as a terminal leukaemic transformation.

Results

The *peripheral blood* data of the three patients are given in Table 21.

TABLE 21. *Clinical and haematological data in three cases of Pancytopenia.*

Case No.	Sex	Age (yr)	Presumptive etiology	Time relation to start of therapy ^a	Peripheral blood findings					Duration of therapy ^a (mo.)	Outcome related to time of diagnosis
					Haemoglobin (g%)	Reticulocytes (%)	Leucocytes (/mm ³)	Granulocytes (/mm ³)	Platelets (/mm ³)		
1. J.M.	M	11	Unknown	a. Immediately before	6.1	2.0	2,300	500	<10,000	3	Died from haemorrhage 6 mo. after
				b. Two months after	7.4	0.2	1,700	200	<10,000		
2. E.T.	F	3	Chloramphenicol	Immediately before	3.4	4.2	3,200	1,000	<10,000	12	Asymptomatic 36 mo. after
3. Y.H.	F	7	Unknown (chloramphenicol?)	Two months after	6.4	3.6	2,000	800	<10,000	3	Died from haemorrhage 9 mo. after

^a Testosterone and corticosteroid [101].

These data were obtained at the same time as the bone marrow analysis and reveal in all cases a severe pancytopenia.

The *bone marrow* findings of each case are summarized in Table 22. The *differential counts* had certain features in common. The 'stem cells' had an apparently normal relative distribution in all marrow aspirates. There was no eosinophilia (neither was there in peripheral blood), and monocytes were not significantly increased in any patient. In most other respects the findings differed from one another, and they are therefore reported separately below.

In *Case 1* there was nearly complete aplasia in both marrow aspirates and

thus the absolute numbers of both the erythropoietic and neutrophil granulopoietic cell lines were markedly reduced. Lymphocytes were the dominant cell type. Many erythroblasts were 'megaloblastoid' with a relatively large cytoplasm and some contained double, triple or quadruple nuclei. Abnormal morphology was noted also in granulocytic precursors with irregular chromatin structure and more intense azurophil granulation. The very few megakaryocytes found in the first marrow aspirate had a dense nuclear structure and a non-granular cytoplasm. An increased frequency of plasma cells was manifest in the first aspirate and in the second was considerably increased.

The bone marrow specimen of *Case*

TABLE 22. *Bone marrow findings in three cases of Pancytopenia.*

Case No.	Cellularity	‘Stem cells’	Erythropoietic cells	Differential count (%)			Monocytes	Lymphocytes	Mega-karyocytes	Labelling index		
				Granulopoietic cells	Neutrophils	Eosinophils				Plasma cells	St	Er
J.M. a.	Severe decrease	9.6	4.1	5.4	0.2	2.6	0.9	72.7	very few	.00	.43	.28
b.	Severe decrease	2.9	5.5	4.7	0	14.3	1.5	70.0	none	.04	.51	.43
E.T.	Moderate decrease	3.9	13.7	30.9	0.7	0.2	4.4	46.0	none	.06	.40	.39
Y.H.	Moderate increase	4.3	33.4	40.1	0.5	0.4	5.0	10.5	moderate increase	.12	.41	.15
Healthy children	—	3—6	5—20	30—65	1—6	0—0.2	1—4	10—40	—	.25—.30	.49+.06	.28±.04

2 had an almost normal appearance except for the absence of megakaryocytes. The proportion of lymphocytes was somewhat increased while neutrophils were at the lower limit of the normal range. A ‘megaloblastoid’ pattern within erythropoiesis was also evident in this case.

The fourth aspirate of *Case 3*, in September 1963, was difficult to interpret. The cellularity was moderately increased. The cells apparently remained not properly mixed in the aspirate even after shaking since the differential counts in different smears showed rather wide divergences. The distribution given in Table 22 is from a smear with a large proportion of erythroblasts. However, the relative proportions of lymphocytes and neutrophil granulopoietic cells appeared normal though there was a marked disproportion between the different neutrophil compartments (see below).

Granulocyte morphology was also irregular with vacuolization and abnormal nuclear shape. The ‘megaloblastoid’ pattern in erythroblasts which had been found in the two previous aspirates was not apparent. Megakaryocytes appeared moderately increased in number and were mostly of immature type, some being vacuolized with pyknotic nuclei. The occasional platelets varied widely in size.

An analysis of the *proportional distribution* of the differentiated cell lines is given in Table 23. With regard to the erythropoietic line, marked divergence from the normal pattern occurred only in *Case 3*, the other two children were not significantly different from normal. The ratio between myelocytes (Gr-2) and promyelocytes (Gr-1) was apparently normal in the first aspirate of *Case 1* (1a) and in *Case 2*. *Case 3* had a markedly reduced proportion of Gr-2, and *Case 1* had

TABLE 23. *Proportional distribution of erythropoietic and granulopoietic bone marrow cells in three cases of Pancytopenia. The size of each compartment is relative to compartment 1 (Er-1 and Gr-1).*

Case No.	Erythropoietic cells						Granulopoietic cells					
	Er-P			Er-NP	NP/P	Cells counted	Gr-P		Gr-NP	NP/P	Cells counted	
	Er-1	Er-2	Er-3	Er-4			Gr-1	Gr-2	3 + 4			
1. J.M. a.	1	6	5	11	0.9	159	1	1.3	2.7	1.2	266	
b.	1	8	12	15	0.7	451	1	0.6	0.8	0.5	131	
2. E.T.	1	9	12	13	0.6	320	1	1.6	8.0	3.0	309	
3. Y.H.	1	3	4	7	1.0	555	1	0.6	0.9	0.6	1,035	
60 healthy children	1	5	13	16	0.8	—	1	1.6	8.3	3.2	—	

changed to a similar abnormal pattern at the time of the second aspiration (1b). In both these investigations mature granulocytes (Gr-3 + 4) were very few, relative to the number of proliferating granulopoietic cells, resulting in very low values of the ratio Gr-NP/P. This ratio was moderately reduced in Case 1a and was normal in Case 2.

The *labelling indices* of different proliferating pools and compartments are given in Tables 22 and 24. The most striking divergence from the normal pattern was the very low proportion of 'stem cells' that incorporated ^3H -thymidine *in vitro*. In Case 1 no labelled 'stem cells' at all were found after incubation of the first aspirate (1a).

TABLE 24. *Labelling indices of erythropoietic and granulopoietic bone marrow cells in three cases of Pancytopenia.*

Case No.	Erythropoietic cells				Granulopoietic cells		
	Er-1	Er-2	Er-3	Er-P	Gr-1	Gr-2	Gr-P
1. J.M. a.	1.00	.41	.33	.43	.31	.25	.28
b.	.78	.84	.30	.49	.47	.38	.41
2. E.T.	.71	.59	.23	.40	.46	.36	.39
3. Y.H.	.26	.60	.32	.41	.18	.11	.15
53 healthy children	.52	.63	.42	.49	.32	.25	.28

TABLE 25. *Mean grain counts of proliferating bone marrow pools and compartments in three cases of Pancytopenia.*

Case No.	'Stem cells'	Erythropoietic cells				Granulopoietic cells		
		Er-1	Er-2	Er-3	Er-P	Gr-1	Gr-2	Gr-P
1. J.M. a.	— ^a	50	49	39	46	33	44	39
b.	38	50	47	31	41	45	38	43
2. E.T.	40	50	49	47	48	50	50	50
3. Y.H.	26	26	37	27	32	42	37	40
53 healthy children	38	44	36	25	30	37	32	34

^aNo labelled 'stem cells' found.

The labelling indices of the *erythropoietic* cells were low in three aspirates (1a, 2 and 3) although within the 95 % confidence limits of the normal material. The labelling pattern of individual compartments (Table 24) showed no significant divergence from normal. The proportion of labelled orthochromatic erythroblasts (Er-3) was, however, low in all aspirates. In Case 3 the very low labelling index of proerythroblasts (Er-1) was calculated from 37 cells and may therefore have some significance.

In the *granulopoietic* cell line the divergence from normal was more obvious, except for the first aspirate of Case 1 (1a) where a quite normal labelling pattern was found. The second marrow specimen of Case 1 (1b) and that of Case 2 showed similar and markedly elevated labelling indices of both pool and individual compartments. In contradistinction, Case 3 had very low indices of both pool and compartments.

Immature plasma cells were found, some of which were labelled, but they were too few to allow any conclusions about their labelling pattern.

Mean grain counts of 'stem cells' and proliferating pools and compartments within erythropoiesis and granulopoiesis are given in Table 25. No significant abnormality was found for 'stem cells' except in aspirate 1a which totally lacked labelled 'stem cells'. The erythropoietic cells in Cases 1 and 2 showed a tendency to high mean grain counts, while Case 3 was normal except for a low value for Er-1. In granulopoiesis most values were not significantly abnormal. There were, however, moderate increases of granulopoietic mean grain counts in Case 2.

Discussion

All three cases had a serious pancytopenia and abnormal bone marrow findings. The latter, however, were not

of uniform type. The marrow was severely hypocellular (Case 1), moderately hypocellular (Case 2), and moderately hypercellular (Case 3). A presumptive etiology was known only in Case 2 (chloramphenicol) who survived, probably as a result of the testosterone and corticosteroid therapy. Cases 1 and 3 died, hormonal therapy being apparently inefficacious. The immediate cause of death was gastrointestinal haemorrhage in both cases. Autopsy revealed a severely hypoplastic marrow in Case 1 and a hyperplastic marrow with leukaemic transformation in Case 3.

Case 1 fulfilled the criteria of 'aplastic anaemia': peripheral pancytopenia, a hypocellular marrow, the absence of hepatosplenomegaly and lymphadenopathy and no history of unusual haematological disorders [36]. In Case 2 there was only moderate hypocellularity but the remaining criteria were fulfilled. The absence of megakaryocytes and the clinical course support the diagnosis of 'aplastic anaemia'. It is possible that another marrow puncture would have shown a more hypoplastic state since the specimen investigated was somewhat heterogeneous and may not have represented a wholly reliable picture of the bone marrow state.

The pathological picture in Case 3 was quite different, being characterized by pancytopenia and a bizarre marrow hyperplasia. This haematological syndrome has been designated pseudo-aplastic anaemia, refractory anaemia with hyperplastic bone marrow, chronic bone marrow failure, etc.

[114]. The etiology is generally unknown and the outcome is usually fatal. A similar state may occur as a 'praeleukaemia stage' of myelo- or monoblastic leukaemia [13, 77], and this was evidently the situation in this case, since the post-mortem diagnosis was 'acute leukaemia'.

The one obvious marrow similarity between all three cases was the significantly low labelling index of the 'stem cell' pool. The relative distribution of 'stem cells' was apparently normal in all aspirates, but the absolute size was certainly reduced in Case 1 and possibly increased in Case 3. The low labelling index means that an abnormally small proportion of the 'stem cells' were in a generative cell cycle at the time of incubation. There are two main alternatives for explaining this:

(1) There is a reduced number of 'stem cells' available for triggering off into the differentiated cell lines. The normal relative size of the 'stem cell' pool is then due to an abnormally prolonged stay in the ' G_0 phase' until the cells finally enter the generative cycle ($G_0 \rightarrow G_1 - S - G_2 - M$) in order to either leave or repopulate the 'stem cell' pool. This might be due, for example, to a raised threshold to differentiation-inducing stimuli.

(2) There is a normal or increased number of 'stem cells' in G_0 phase but some never enter a generative cycle and die in G_0 phase, e.g. due to immunological reactions. The number of proliferative cells is thus reduced, and this in turn reduces the influx into the differentiated pools.

The cell flow into the differentiated lines was below normal, at least in Cases 1 and 2. The labelling patterns and the proportional distributions in the *erythropoietic cell lines* appear rather similar in these two cases. The relatively low labelling indices in Cases 1a and 2 might be due, either to chance, or to disturbances in proliferation and/or maturation (cf. Infantile genetic agranulocytosis). A lengthened pool transit time together with a normal pool distribution means a reduced flow through, and hence a reduced efflux from, the erythroid pool.

In Case 3 proerythroblasts (Er-1) were relatively more numerous compared with the populations of the more mature erythroid compartments. The labelling index of the erythroid pool was low and that of compartment Er-1 was especially low. These features in combination with a low mean grain count of Er-1 indicate an obvious disturbance of cellular proliferation within the first generation(s) of the erythroid pool. Again, the findings may be interpreted as due to prolongation of generation time(s) or to cell death with 'ineffective' erythropoiesis, both alternatives leading to a reduced outflow from the erythroid pool.

Within *granulopoiesis*, the similarities and differences between different cases are somewhat puzzling. The first aspirate of Case 1 (1a) showed a normal proportional distribution within the proliferating pool and a normal labelling index. The non-proliferating pool was relatively small and this may partly have been due to an earlier than normal release of mature granulocytes,

i.e. a very small marrow granulocytic reserve existed. The main reason for the peripheral agranulocytosis in Case 1 appeared to be a very much reduced bone marrow production on account of a reduced influx from the 'stem cell' pool. In Case 2 the findings were similar except for a high labelling index. Whether this was due to a shortened pool transit time or to a moderate loss of cells in S phase or merely due to chance was not established. In the second aspirate of Case 1 (1b) and in Case 3 there was an abnormal, reversed ratio between Gr-2 and Gr-1 and a very low proportion of non-proliferating granulocytes (Gr-3 + 4). These findings indicate 'ineffective' granulopoiesis with considerable loss of cells by death in the proliferating pool. In order to produce such a distribution pattern, a 'cell flow' with no losses due to death would imply a markedly prolonged Gr-1 transit time and a very shortened Gr-2 transit time. This is not compatible with the uniform trend of labelling indices found in each case, i.e. high in both compartments of Case 1b and low in both compartments of Case 3. The labelling indices of Case 1b were calculated from a rather low number of cells because of the infrequency in the smears, and whether the cell loss occurs in the S phase, thus raising the index, or in G_1 or G_2 cannot be decided from these data. The calculations in Case 3 are more significant due to the larger number of cells counted. The low indices and the distribution pattern found in this case indicate most probably a high proportion of cell death in G_1 phase.

This study of Pancytopenia includes three cases. Two of the cases were diagnosed as 'aplastic anaemia' with varying degrees of bone marrow hypocellularity. The findings in these two cases have many features in common and the following hypothesis of the pathogenetic mechanism is proposed: The bone marrow insufficiency is mainly due to a reduced flow from the 'stem cell' pool to the differentiated pools. This is indicated by reduced proliferation in the 'stem cell' pool and a small number of cells in the first compartments of the differentiated cell lines. The absolute size of the 'stem cell' pool is probably reduced in a hypoplastic marrow. This means that the self-perpetuating feed-back mechanism is severely restricted. The subsequent proliferation and maturation in the erythropoietic and granulopoietic cell lines are not markedly abnormal. The very low number of megakaryocytes is in accord with the concept of a non-specific or quantitative disturbance due to a 'stem cell' defect which affects all differentiated lines. Bernard and Najean in their study of 102 cases of 'Idiopathic pancytopenias' [10] concluded that the defect within erythropoiesis appeared to be quantitative. They made no evaluation of the granulocytic cell series.

The third case had a somewhat

hyperplastic marrow with a terminal leukaemic transformation. In common with the other two cases the proliferative activity of the 'stem cell' pool was reduced but there were also evident signs of disturbed proliferation and maturation within erythropoiesis and granulopoiesis. The increased frequency of abnormal megakaryocytes also indicated a qualitative disturbance. Within erythropoiesis the findings were interpreted as indicating 'ineffective' erythropoiesis with cell death mainly affecting the most immature erythroblasts. The much depressed release of granulocytes from the marrow was due partly to a reduced influx from the 'stem cell' pool and also to 'ineffective' granulopoiesis, cell death probably occurring in G_1 phase.

The finding of reduced proliferation in immature undifferentiated cells in the bone marrow of all three cases of Pancytopenia appears to justify their classification as 'stem cells'. Their kinetic behaviour fits with the theory of a multipotent 'stem cell' pool, the outflow from which is the main regulator of the production of differentiated bone marrow cells. In Case 1 with a severely hypoplastic marrow, lymphocytes were the predominant cell type. Whether their relative high frequency indicates a connection between 'stem cells' and lymphocytes cannot be evaluated.

Summary

The aim of the present work was to study certain aspects of the kinetics of bone marrow cell proliferation in normal children and in children with haematological disease. In addition to haematological techniques the following methods were used with bone marrow aspirates: semiquantitative assessment of the cellularity, differential cell counts, and study of the proliferative activity of the cells by means of *in vitro* one hour incubation with tritiated thymidine and stripping film autoradiography. Labelled thymidine incorporation into nuclear DNA was estimated by determining the distribution of developed silver grains over the cells (grain count). The relative number of labelled cells (labelling index) is a measure of the proliferative activity.

Chapters I and II comprise a description and critical discussion of the methods used. Some technical modifications were introduced in order to improve the reproducibility of the methods. The use of the brush technique for making smears was shown to give a reasonably uniform distribution on the smear of all cell types, except megakaryocytes, and a low percentage of disintegrated cells. The introduction into the classification system of a

cell group designated '*stem cells*' was proposed. This group conforms to the following morphological criteria and labelling behaviour: (a) the cells are mononuclear with a finely reticular chromatin and usually visible nucleoli, i.e. are *immature*, (b) they lack definite characteristics of any differentiated cell line, i.e. are *undifferentiated*, and (c) they have the capacity to incorporate thymidine, i.e. are *proliferative*. The proliferating and non-proliferating pools were defined in the erythropoietic and granulopoietic cell lines on the basis of their labelling behaviour.

Chapter III describes the findings in 60 healthy infants and children, aged from a few hours to 8 years, special attention being paid to the situation during the first month of life, and more especially to the first week. The results of the differential counts confirmed and extended the results of previous investigations. 'Stem cells', as defined above, were present in all smears in significant proportions, the range of variation being relatively small. During the first week of life the labelling index of the 'stem cell' pool was low but then gradually increased, attaining at about one month a value which then remained fairly constant. The labelling indices within

the erythropoietic and granulopoietic pools were quite constant throughout the age period and did not differ significantly from the results of comparable investigations in adults. The ratio between non-proliferating and proliferating erythroblasts, however, decreased during the first month, after which it remained essentially constant. These findings were interpreted as indicating a rapid neonatal decrease in erythropoietic activity during this age period and confirm the results of previous investigators, who came to the same conclusion using other methods. Furthermore the results of the present study indicate that the decrease is due to a reduction in cellular proliferation at the 'stem cell' level.

Chapter IV describes studies of: A. Haemolytic disease of the newborn; B. Congenital hypoplastic anaemia; C. Infantile genetic agranulocytosis; and D. Pancytopenia.

A. Five neonates with haemolytic disease due to incompatibility within Rh-groups between mother and child were investigated during the first day of life and before any exchange transfusion had been made. The only abnormal finding was that the labelling index of the 'stem cell' pool was considerably higher than that of healthy neonates. Haemolytic disease of the newborn is thus characterized by an increased proliferative activity of the 'stem cell' pool and an apparently normal proliferation and maturation within the medullary erythropoietic cell line.

B. Three infants with Congenital (erythroid) hypoplastic anaemia were

investigated prior to corticosteroid therapy. The bone marrow differential counts were normal except for low numbers of erythroblasts, the deficiency being greater in the more mature compartments. The labelling indices of the 'stem cell' and granulopoietic pools were normal except for a low index of the 'stem cell' pool in one of the cases. The labelling index of the erythropoietic pool was apparently subnormal in two cases and normal in one. The findings were interpreted as indicative of a low influx from the 'stem cell' pool to the erythroid cell line and 'ineffective' erythropoiesis, i.e. cell death, within the erythron. The pathophysiological disturbances appear to be those expected from either a deficiency of erythropoietin or a blocking of its effect, the latter being more likely, as an elevated serum erythropoietin level is a frequent finding in this disease. This would imply that erythropoietin is essential for both transformation of 'stem cells' and development of differentiated stages.

C. Three children with Infantile genetic agranulocytosis were studied, one of them with repeated bone marrow aspirations during the age period two weeks to six months. In all cases there was a severe neutropenia and a varying degree of monocytosis in the peripheral blood. The bone marrow differential counts showed a marked reduction of myelocytes and mature neutrophils. 'Stem cells' and erythroid elements were normal except for one low value of the 'stem cell' labelling index in one of the cases. The labelling

indices of the granulopoietic compartments showed a tendency to low values in myelocytes and close to normal values in promyelocytes. These findings were interpreted as indicating that the transformation of 'stem cells' to promyelocytes was normal, but that there was a destruction of cells in later stages.

D. Three cases with pancytopenia were investigated. Two of the cases showed many features in common: bone marrow hypocellularity, a very low number of megakaryocytes, a low 'stem cell' labelling index, and no marked signs of disturbances in the develop-

ment of the erythropoietic and granulopoietic cell lines. These findings were interpreted as indicating that the bone marrow insufficiency was mainly due to a reduced flow from the 'stem cell' pool to the differentiated cell lines. The third case showed a hypercellular bone marrow with abundant megakaryocytes and very abnormal distribution within the differentiated cell lines but also a low 'stem cell' labelling index. In this case, the pancytopenia seemed to be due, mainly to destruction of cells during maturation and only to some extent to reduced influx from the 'stem cell' pool.

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Case No.	Age			Differential count (%)												Labelling index								
				Erythropoietic cells				Granulopoietic cells				Eosinophils												
	Stem cells	Er-1		Er-2		Er-3		Er-4		Gr-1		Gr-2		Gr-3		Gr-4		Mature	Immature	Basophils	Plasma cells	Monocytes	Lymphocytes	Disinteg. cells
		Year	Month	Day																				
1	0	0	1	25	5	35	57	157	17	102	110	334	17	41			12	58	44	.12	.53	.29		
2	0	0	1	68	8	51	148	239	26	68	31	213	2	7			5	208	42	11	42	37		
3	0	0	1	67	8	36	63	119	40	47	44	389	7	11			13	128	52	11	37	30		
4	0	0	2	86	2	13	49	112	50	74	56	412	13	39			26	102	31	10	46	27		
5	0	0	2	50	1	16	30	38	28	44	19	439	2	26			1	48	7	12	50	32		
6	0	0	3	69	7	21	44	43	74	102	45	458	9	22			3	80	31	12	42	31		
7	0	0	3	159	4	9	22	41	79	72	22	438	8	45			15	69	28	10	47	31		
8	0	0	4	98	1	8	37	64	69	83	65	353	19	44			15	101	24	08	38	28		
9	0	0	4	74	6	10	21	58	119	122	47	419	9	30				104	35	07	46	23		
10	0	0	5	89	5	36	43	61	69	112	68	392	19	18			5	95	36	22	49	25		
11	0	0	5	56	4	15	9	14	98	74	38	541	10	30	2		4	63	59	09	54	32		
12	0	0	6	90	1	10	21	26	34	61	28	488	10	24			32	159	19	14	50	31		
13	0	0	7	82	1	4	11	18	38	83	58	516	6	23			18	134	31	11	48	29		
14	0	0	7	159	1	6	14	24	96	58	31	424	15	29			16	93	38	06	58	21		
15	0	0	9	100		34	70	80	117	108	32	332	26	24				46	32	21	46	21		
16	0	0	10	86	5	12	13	20	70	64	51	519	7	22			15	88	30	15	50	25		
17	0	0	10	115	2	32	43	87	117	163	92	292	14	16			10	43	34	20	49	17		
18	0	0	11	54	2	9	17	27	25	52	26	555	6	23	2		19	121	28	24	55	31		
19	0	0	12	132	2	14	25	42	106	59	23	442	40	35			9	36	42	18	52	28		
20	0	0	12	155	5	13	30	46	89	37	33	300	10	40			22	172	44	15	44	25		
21	0	0	13	160		17	34	50	73	69	34	296	17	46			9	205	33	12	46	26		
22	0	0	16	117	4	10	23	24	29	62	31	248	18	23	4		45	289	100	31	57	26		
23	0	0	16	68	5	3	13	22	23	44	39	465	7	27	2	4	70	190	106					
24	0	0	18	89		4	13	12	35	58	39	223	14	53			32	313	48	21	52			
25	0	0	22	138	3	5	6	16	18	71	48	348	7	15			12	294	65	38	50	25		
26	0	0	24	136	9	19	25	34	11	43	31	341	11	51			15	350	12	40	43	26		
27	0	0	25	81	1	6	12	19	19	59	63	210	9	25			28	418	78					
28	0	0	26	71	4	10	28	33	33	95	65	354	6	38	10		31	185	67					
29 ^a	0	0	27	71	3	8	14	20	64	124	74	354	24	26	3	1	26	178	223					
30	0	0	28	151	2	8	17	38	103	86	44	146	14	11			387	17		33	57	26		
31 ^a	0	0	30	94	5	16	32	51	32	66	52	175	10	12			27	415	113					

Case No.	Age			Differential count (%)												Labelling index																		
				Erythropoietic cells				Granulopoietic cells				Eosinophils																						
				Neutrophils				Basophils				Plasma cells																						
				Stem cells	Er-1	Er-2	Er-3	Er-4	Gr-1	Gr-2	Gr-3	Gr-4	Mature	Immature																				
Year	Month	Day	Gr		Er	St	Disinteg. cells	Lymphocytes	Monocytes	Plasma cells	Basophils	Eosinophils	Lymphocytes	Monocytes	Plasma cells	Basophils	Eosinophils	Mature	Immature															
																				Gr	Er	St	Disinteg. cells	Lymphocytes	Monocytes	Plasma cells	Basophils	Eosinophils	Lymphocytes	Monocytes	Plasma cells	Basophils	Eosinophils	Mature
				Gr																														
Gr	Er	St	Disinteg. cells		Lymphocytes	Monocytes	Plasma cells	Basophils	Eosinophils	Lymphocytes	Monocytes	Plasma cells	Basophils	Eosinophils	Mature	Immature																		

| 32 | 0 | 1 | 15 | 161 | 2 | 6 | 37 | 34 | 44 | 77 | 43 | 329 | 9 | 8 | |
^a Coverslip smear

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Abbreviations

DNA	deoxyribonucleic acid
M	mitosis
G ₁	postmitotic resting period
S	DNA synthesis period
G ₂	premitotic resting period
³ H	tritium
ARG	autoradiography
L. I.	labelling index
Diff. %	differential count (%)
S. D.	standard deviation
S. E.	standard error of the mean
ESF	erythropoietin

St	'stem cell'
Er-1	proerythroblast
Er-2	basophil erythroblast
Er-3	polychromatic erythroblast
Er-4	orthochromatic erythroblast
Er-P	proliferating erythropoietic pool
Er-NP	non-proliferating erythropoietic pool
Gr-1	promyelocyte
Gr-2	myelocyte
Gr-3	metamyelocyte
Gr-4	polymorphonuclear granulocyte
Gr-P	proliferating granulopoietic pool
Gr-NP	non-proliferating granulopoietic pool

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